Does Size Matter?

Study of allometry of reproductive organs in selected populations of *Drosophila melanogaster*.

Tejinder Singh Chechi

A dissertation submitted for the partial fulfillment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2015

Dedicated to my parents

for letting me ‘fly’...

Certificate of Examination

This is to certify that the dissertation titled **Does size matter? Study of allometry of reproductive organs in selected populations of *Drosophila melanogester*,** submitted by **Tejinder Singh Chechi (Reg. No.MS10054)** for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Declaration

The work presented in this dissertation has been carried out by me with **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.

Tejinder Singh Chechi

(Candidate)

Dated: April 24, 2015

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)

Acknowledgement

I thank all living and non-living entities who have made this thesis possible, from the bottom of my heart.

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Notations

M – Male biased selection line

C- Control population

F- Female biased selection line

ANOVA- Analysis of variance

ACPs- Accessory Gland Proteins

ABSTRACT

Males from populations which face higher degree of intersexual conflict and sexual selection have increased sperm defense ability. Increased sperm defense ability might have a anatomical and physiological basis in terms of increased testis and/or accessory gland size along with altered patterns of ejaculate investment. To test this hypothesis, we quantified the size of reproductive organs of male *Drosophila melanogaster* selected for different levels of sexual selection.

We measured the testes area and the accessory gland area of the flies selected for low and high levels of sexual conflict. Measurements were done on virgins and after a single mating. The difference in the size of these organs pre and post mating is used as an indicator of the quantity of ejaculate investment. Contrary to some previous studies, we found no significant difference in the testes area or the accessory gland area between selected and control populations either in virgin or mated conditions. Mating decreased accessory gland area but not testis area. This indicates that the fitness advantage of males from high conflict populations might result from differences in accessory gland proteins and sperm quality.

INTRODUCTION
CHAPTER 1

Sexual selection is the evolutionary process that favors the increase in frequency of genes that confer a reproductive advantage (Pizzari *et al.* 2002). In polyandrous species, where females can have multiple matings, sexual selection persists after copulation to the point of fertilization. The postcopulatory sexual selection results in sperm competition among the males to fertilize the ova of a female (Parker 1970).

Sperm competition is a widespread phenomenon influencing male anatomy and physiology. Sperm competition theory (Parker 1970) predicts that when competition is high, individuals tend to increase reproductive investment. This often leads to relative increase in sperm number. Correlation studies both within and across the species indicate that increased testes size results in production of more sperms (Harcourt *et al*. 1981 ; Kenagy&Trombulak 1986; Gage 1994; Hosken 1997; Stockley *et al*. 1997 ; Simmons *et al*. 1999). Direct relation between sperm competition and gonad size has been established in many organisms for example fishes (Møller*et al.* 1997), insects (Hosken *et al.* 2001) etc. Fruit flies have been widely used to study sperm competition and its correlated effect on anatomy of male gonads. In Drosophila, male gonads not only consist of a pair of testes but along with that they contain a pair of accessory glands. These accessory glands produce more than 100 different kinds of proteins which are transferred along with sperms in ejaculate. In postcopulatory sexual selection, accessory gland proteins (ACPs) play an important role in sperm competition and ensuring fertilization success. Along with facilitating sperm transfer, ACPs exert wide-ranging effects on female reproductive activity and they improve the male's chances of siring a significant proportion of the female's offspring. The accessory gland secretions may affect virtually all aspects of the female's reproductive activity (Fleischmann *et al.* 1991). The secretions may render her unwilling or unable to remate for some time (Chapman *et al.* 1996), facilitating sperm storage and ensuring that any eggs laid will be fertilized by that male's sperm. They may stimulate an increase in the number and rate of development of eggs and modulate ovulation and/or oviposition (Ashburner, 1989; Chapman

1996). Linklater et al. (2007) showed that higher sperm competition had no effect on testis size.These results were contradictory to the findings of Ward et al. (2001) which showed larger testis size in populations of dung beetles evolved under higher level of sperm competition. However Linklater et al (2007) did find a faster decrease in accessory gland size of males under increased risk of sperm competition upon multiple matings with virgin females compared to males under decreased risk of sperm competition, indicating a greater ejaculate depletion rate. Even though there exists some evidence that sexual conflict has an effect on male gonad size, there is no consensus.

Different level of sperm competition is often positively correlated with differential levels of mate availability(Wigby and Chapman 2004; Michanczyk *et al.* 2010) . For males, under high sperm competition, access to females (and thereby frequency of matings) is often less, as compared to that under lower sperm competition. In such a case, to ensure success in post-copulatory sexual selection, males facing higher sperm competition would invest more in each mating. This is predicted to result in evolved sperm depletion as well as ACPs depletion pattern in the populations with different levels of sexual selection.

We subjected this idea to test in populations of *Drosophila melanogaster* evolved for different levels of sexual conflict obtained by varying operational sex ratio. We used two regimes in this study- in one of them the sex ratio was male biased (♂:♀ = 3:1), in the other it was female biased (♂:♀ = 1:3). At the time of the study, the populations had evolved under these conditions for more than 135 generations. Previous results from these populations indicate that males from male biased regime (higher sexual conflict) are under higher risk of sperm competition and they have evolved higher sperm competitive ability compared to males from female biased regime (lower sexual conflict).

Results also show that males from male biased regime have higher copulation duration and mating success. (Nandy *et al.* 2013), thus having fitness advantage over males from female biased regime.This indicates that males facing high sperm competition would have to invest more in sperm production/quality to ensure fertilization success. On the other hand, males facing relatively low sperm competition would at least not increase investment in sperm production/quality.

This fitness advantage gained by males facing relatively high sperm competition can result from increased investment in ejaculate production and/or the improved quality of ejaculate.Since sperms and ACPs are the two primary ingredients of the ejaculate in fruit flies, evolution of increased sperm competition in male biased populations could be a result of (a) change is sperm quality, quantity etc. (b) change in ACP production or both. This could result from the increased investment in ACPs production, correlating to increased accessory gland size.

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With this background and results, the questions we were interested in investigating were as follows:

a) Is there a physiological basis to the fitness advantages that males from relatively higher sexual conflict/sperm competition have, in terms of difference in testes size and accessory gland size?
b) Has the sperm and ACPs depletion pattern evolved in the populations selected for different levels of sexual selection?

GENERAL EXPERIMENTAL METHODS
CHAPTER 2

2.1 Experimental Population

Drosophila melanogaster is a holometabolous dipteran insect, belonging to the family Drosophilidae. Their life cycle passes through four distinct stages – egg, larva, pupa and adult. Under the standard laboratory conditions-25^{o}C temperature, 60-90% relative humidity, their metamorphosis (as seen in laboratory adapted populations maintained at 25°C and moderate larval density) follows the pattern described here in brief (Figure 2.1). Eggs usually take around 18 hours to hatch. The larval stage passes through three sub-stages or instars – first, second and third. During larval stage they dig into the food (the fly media in case of laboratory cultures) and actively feed upon the available food. Upon reaching a "critical size" they become committed to the post larval development. The late third instar larvae stops feeding, comes out of the food and becomes stationary on some suitable substrate (vial/bottle wall or the cotton plug for laboratory cultures) and forms pupae by secreting a chitinous covering. Larval stage lasts for approximately 4-5 days. Pupal stage lasts for about 4-5 days.Adult flies come out of the pupal shell – a process commonly referred to as 'eclosion'. The entire preadult development takes about 8-10 days. The adult males and females do not eclose as reproductively active individuals. They usually take 8-10 hours to become reproductively mature.. Females might start laying eggs by 24 hours of eclosion.

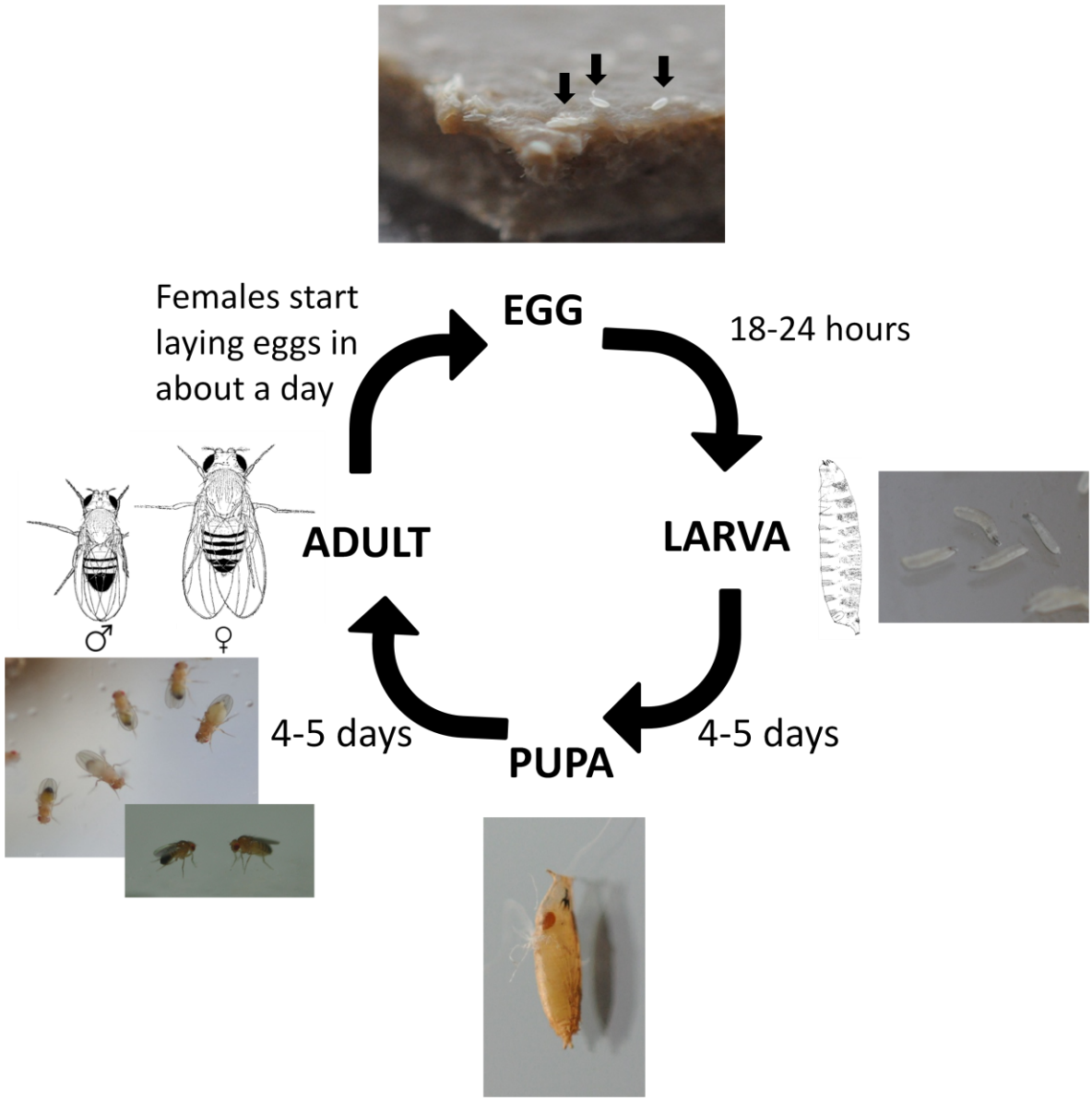


Figure 2.1: Life cycle of fruit flies (*Drosophila melanogaster*). Generation time corresponds to the typical fly life cycle under laboratory condition (25^{o}C temperature, 60% RH).

Laboratory adapted population: LH and derivatives

The base population LH (named after the original founder, Larry Harshman) was founded with 400 wild caught females from central California, USA in 1991 (Chippindale and Rice 2001). LH_{st} was derived from LH base population by introducing the reseccive-autosomal trait scarlet-eye ('st') by repeated back crossing (Prasad et al. 2007). $LH_{st}$is maintained under similar environementalconditions.LH_{st} is periodically backcrossed with LH to maintain the genetic uniformity across the two populations.

Sex Ratio Selection-line: Experimental evolution of populations under altered operational sex ratio

The work of this thesis was carried out on a set of *D. melanogaster* populations subjected to experimental evolution under different operational sex ratio (ratio of males to females available for reproduction). The aim was to look into the role of interlocus sexual conflict on the evolution of behavioural and life-history traits in these populations. Different operational sex ratio is thought to generate different levels of male-male competition and interlocus sexual conflict in a population. Male biased sex ratio is expected to be a more competitive environment for the males, and together with increased opportunity of male interactions per female, is thought to generate 'high' conflict condition. Equal sex ratio is the standard ancestral condition. Females biased sex ratio on the other end is thought to relax intersexual conflict and male-male competition. This method of varying the level of interlocus conflict was adopted in previous studies as well but with several differences (Wigby and Chapman 2004, 2006, Linklater et al. 2007).

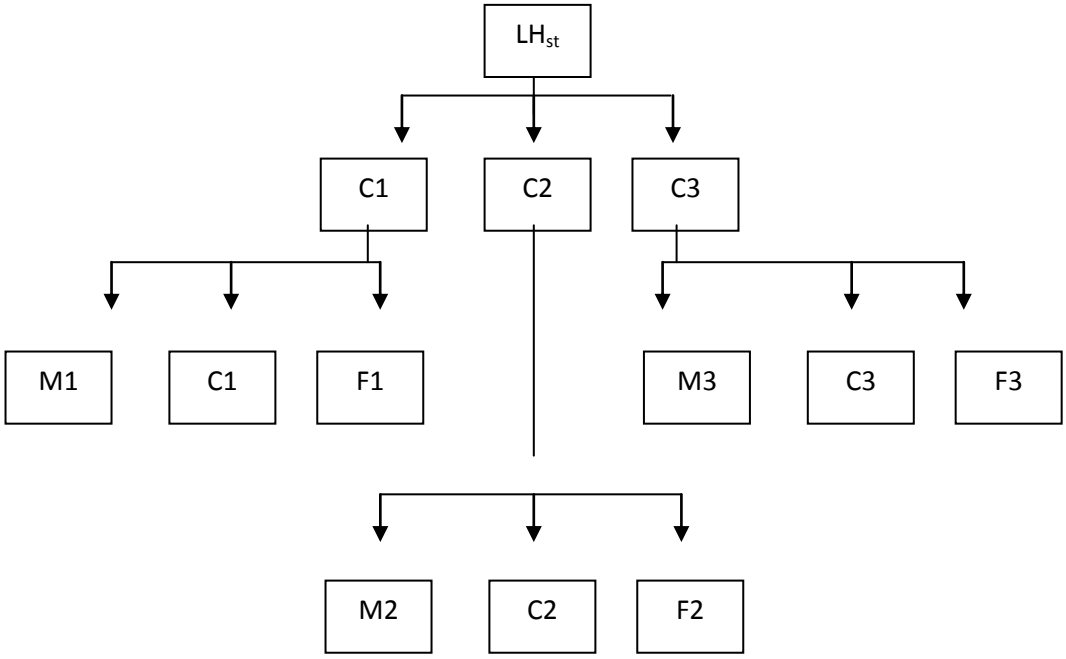
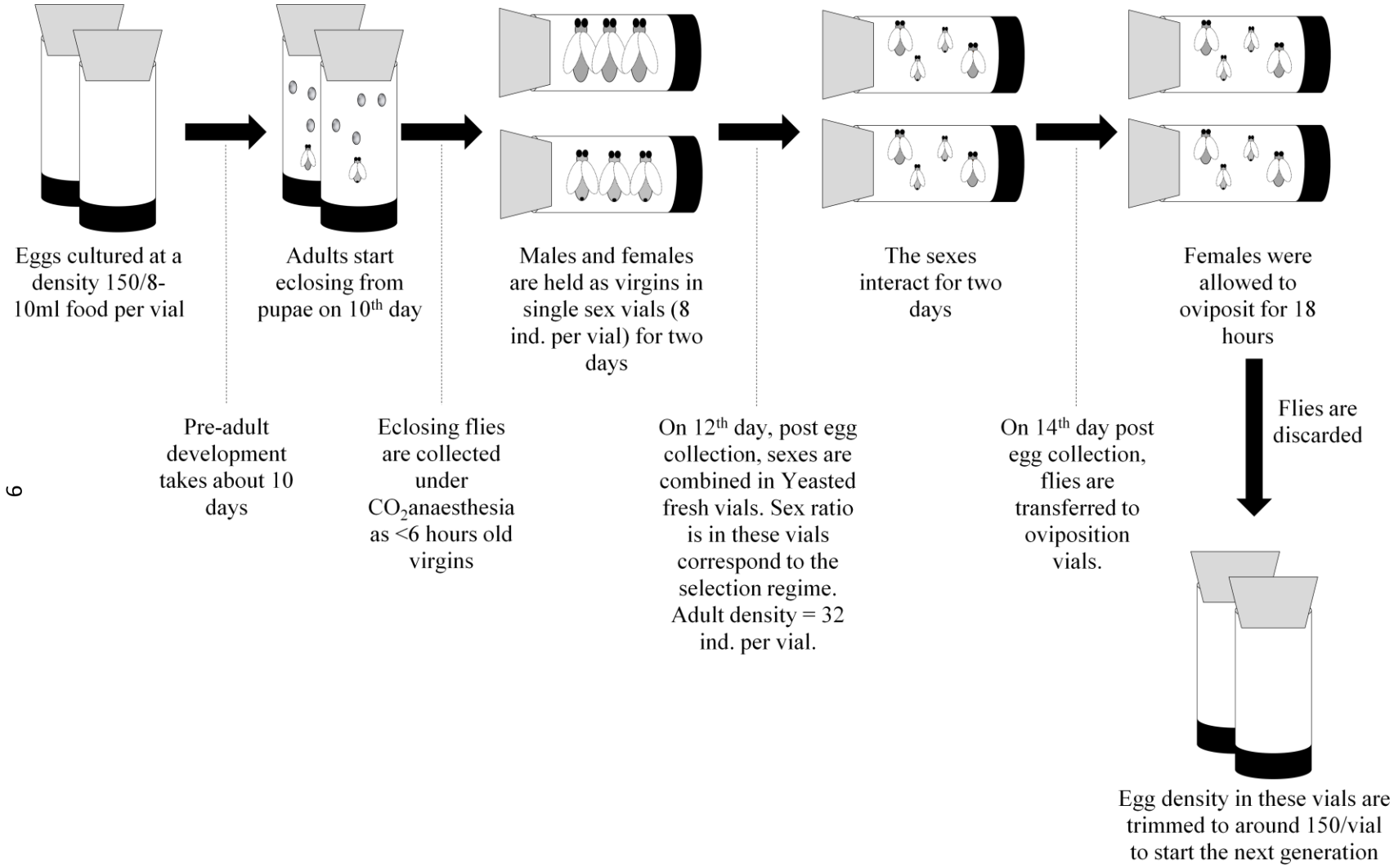


Figure 2.2: The 9 selected populations were created ultimately from LH_{st} baseline. LH_{st} was first used to create three replicate populations - C1, C2 and C3. These three populations were maintained for 5 generations and then each of them were split into three sex ratio regimes - male biased (M), female biased (F) and the ancestral equal sex ratio (C). Populations bearing same numeric subscript share common ancestry. For example, M1, F1 and C1 share common ancestor (i.e. C1).

Derivation and maintenance

Three replicate populations, names C1-3, were derived from the LH_{st} base population and maintained for five generations under equal sex ratio (and other conditions described later). Then each replicate was used to derive two additional regimes – male biased (M1-3) and female biased (F1-3). Thus after six generations of splitting from the base populations,nine populations – three sex ratio regime, each with three replicates, were established. Thus, populations bearing the same numerical subscript share a common ancestry (Figure 2.2) and are more closely related to each other compared to populations bearing different numerical subscripts. For example, M1 is more closely related to C1 and F1 than to M2. Additionally, during regular maintenance, replicate populations bearing the same numerical subscript are always handled together. Hence replicates bearing the same numerical subscripts are treated as statistical 'Blocks' in the analysis. The whole experiment consists of three statistical blocks (Blocks 1, 2 and 3). All aspect of the maintenance regime was kept equal across the regimes except the adult sex ratio. All the populations are maintained as 14-day discrete generation cycle (Figure 2.4), under 25^{o}C temperature, 60-80% relative humidity and 12-hours light / 12-hours dark. The maintenance regime of the populations is described in Figure 2.4. Every generation eggs are cultured in food vials under moderate density (140-160 eggs / 8-10ml of food in 8-dram vials). The flies take about 10 days to complete the preadult development. On 10^{th} day adult flies star eclosing out of pupae. Just prior eclosion the pupae become dark in appearance indicating the eclosion phase. The adult flies are collected as very young (< 6 hours post eclosion) virgins and held in single sex vials (pre-reproductive vials). The adult flies take around 8-10 hours to become reproductively mature.



Firgure: 2.3 : General maintenance protocol for the selected populations (M1-3, C1-3, and F1-3)

Therefore by this method of collecting the adult flies soon after eclosion ensures their virgin status. Virgin flies are held at a density of 8 flies per vial in the pre-reproductive vials. After two days, on 12^{th} day post egg collection, the sexes are combined in food vials provided with measured amount of live Yeast (adult competition vials). The sex ratio in the adult competition vials were maintained according to the selection regime – male biased (24 males : 8 females) for M-populations, equal sex ratio (16 males : 16 females) for C-populations and female biased (8 males : 24 females) for F-populations. The amount of Yeast available to each female is controlled at 0.467mg. In the adult competition vials, the flies are allowed to interact for two days before transferring them to oviposition vials. Oviposition vials are provisioned with 8-10ml of food. Flies are given a window of 18 hours to lay eggs. After this 18 hour gap, flies are discarded and the egg density in the each vial is trimmed to around 150 per vial to start the next generation. The effective population size was controlled at around 450 for each these 9 populations. Calculation of N_e was done following Crow & Kimura (1970), $N_e = 4N_mN_f/(N_m+N_f)$(Nandy *et al.* 2011; Nandy *et al.* 2013).

2.2 Standardization of and Generation of Experimental Flies

Selection pressure is relaxed for one generation in M and F populations and they are maintained in the baseline population condition, to equalize genotype – environment interactionslike maternal effects. This process is called standardization and the flies thus generated are called standardized flies.

For generating experimental flies, 14 day (from egg collection) old adults from M and F populations were transferred to two cages each containing ~500 flies in a roughly 1:1 rex ratio.A small plate of cornmeal-molasses food with abundant yeast was provided to trigger egg-laying and a moist piece of cotton was provided to prevent desiccation. After 48 hours a fresh plate was given to them, and within 18 hours, eggsat a density of 150 eggs per vial were collected and transferred to 8 dram vials, containing 8-10 mL of cornmeal-molasses food. Ten such vials for each of the selection regimes were set up per block and adults emerging from there were used as experimental flies.

Along with M and F population, 10 vials of egg collection for LH-st population were done in the same manner as mentioned above.

2.3 Experimental Procedure Standardization

The virgin male flies from M and F populations were transferred into empty 8 dram vials on 12^{th} day and frozen at -20^{o}C. Next day, the flies were taken out for dissections, but the flies had become brittle due to lack of moisture and thus dissections became difficult. So to standardize the procedure, the egg collection was repeated and this time the male flies on 12^{th} day were frozen in 8 dram vials which contained 2-3 ml of cornmeal-molasses food, to get rid of the problem of flies becoming brittle.

The dissections were done in PBS solution, to maintain the salinity of body fluid conditions. After trying out many quantities of PBS solution, 9 µL of 1x PBS solution per dissection was standardized.

2.4 Dissections

Frozen flies (at -20^{o}C), after 2 days were taken out for dissection. Using ZEISS steni microscope fitted with a AxioCam ICc1 camera camera both wings, testes and accessory glands were dissected out and images were captured.

Wings

From each fly, both the wings were removed. Using the AxioCam attached to the microscope, image of each wing at fixed 5 x magnification were captured. Wing length is used as a proxy for body size and thus used to normalize the testes and accessory gland area across the M and F populations.

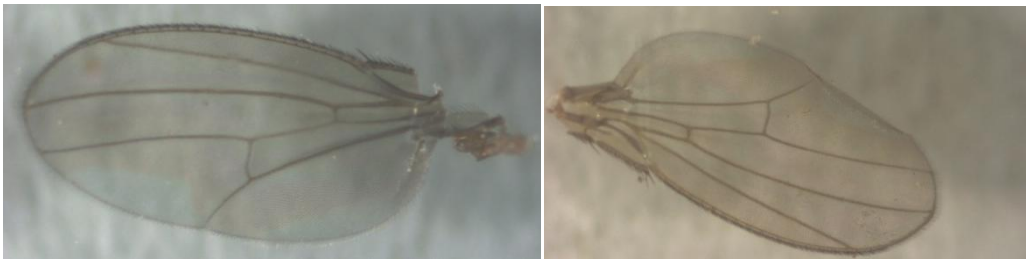


Fig. 2.4: Images of wing taken using ZEISS AxioCam at 5x magnification

Thorax

Picture of thorax was captured from each fly at fixed 3.5 X magnification, using AxioCam camera attached to the microscope. Thorax length is used as a proxy for body size to normalize the testes and accessory gland data across selection regimes.

Fig 2.5: Image of Thorax taken using ZEISS Axiocam at 3.5x magnification

Testes and Accessory Glands

Testes dissection was done in 9µL of 1 X PBS solution. The reproductive organs (Testes and accessory Glands) are present towards the last segments of the abdomen. Using the Forcepand micro-needle, the last segment is pulled out and along with it the internal organs are pulled out. The debris of fats and other organs is cleared from around the testes and accessory glands.

Using the AxioCam attached to the microscope, testes and accessory gland pictures were captured at 5 X magnification.

Fig 2.6: Testes image taken using ZEISS Axiocam at 5x magnification.

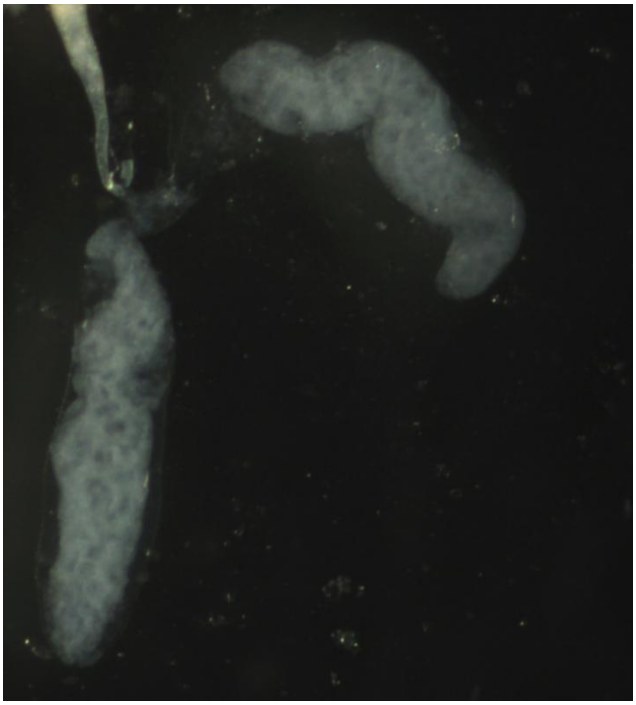


Fig 2.7: Accessory Gland image taken using ZEISS Axiocam at 5x magnification.

2.5 Image Analysis

All the images were analyzed using Image J 1.48. Length was defined using the image of stage micrometer at fixed magnification.

The wing length was calculated using the length tool. Two fixed point were selected and length was measured between those points, for each wing.

For the cross section area of testes and accessory glands, a combination of different tools was required:

a) The brightness and contrast values for each image were adjusted to bring out the subject of picture (Testes or accessory glands) from the background.
b) Using the Threshold adjustment, the area of the subject was detected by the software.
c) Using the wand tool, the area to be measured was marked and measured.

2.6 Data Analysis

Statistical analyses was done usingmixed model analysis of variance (ANOVA) with selection regimes and/or treatment as fixed factors and block as random factor. Statistica 5.0 was used to perform all statistical analyses.

EXPERIMENTAL SETUP AND PROCEDURE
CHAPTER 3

3.1 Experiment 1

The study of allometry of reproductive organs of virgin male Drosophila melanogaster, selected for different levels of sexual conflict.

3.1.a Experimental Setup

The M and F population flies were standardized to get rid of the non-parental effects and egg collection was done from these standardized flies(see chapter 2). For each population, 10 vials of eggs with density of 150 eggs per vial, were collected and transferred to 8 drams vials containing 8-10 ml cornmeal molasses food. On day 9-10 the male flies were collected at the interval of 6 hours to ensure virgin males. for each population, 10 vials with 8 males per vial were collected in 8 dram vial containing 3-4 ml of cornmeal molasses food. They were left for 2 days in the vial to become reproductively mature. On day 12, male flies were frozen at -20^{O}C. This procedure for repeated for all the 3 blocks of M and F populations.

The frozen flies were then dissected to measure the area of testes and accessory glands, normalized by the proxy for body size, it being wing length or thorax length. Dissections were done under the microscope attached with an ZEISS AxioCamICc 1. Images were captured using the AxioCam vision software.

From each block, 15 flies from each population were randomly chosen and dissected out to measure the wing length, testes area and accessory gland area.

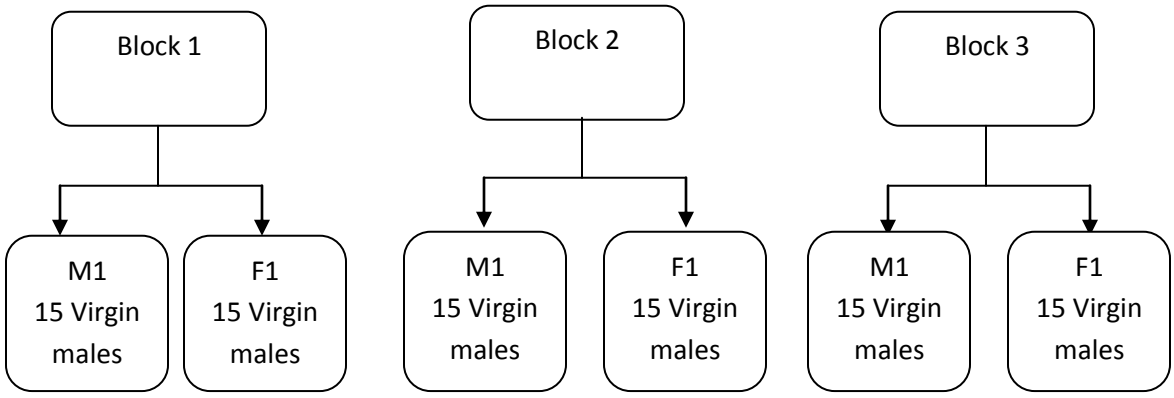


Fig 3.1.1: Sample size of 15 males each from M and F population in each block. Experiment repeated over three blocks.

3.1.b Wing length measurement

Wing length was measured as a proxy for the body size. Both the wings were removed from the fly and images were captured. All the 180 images (3 block x 2 populations x 2 wings x15 flies) were taken at 5 X magnification and length was measured using Image J length tool. All the measurements were averaged out over three independently repeated measurements, to account for any measurement error.

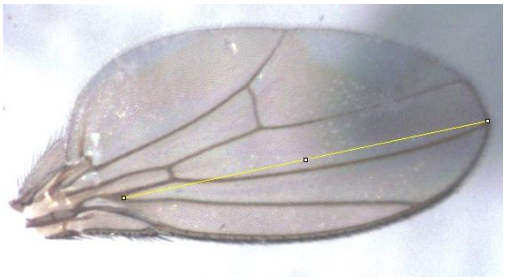


Fig 3.1.2: Points selected for the wing length measurement with length tool marking.

3.1.c Testes and Accessory Gland area

All the 180 images (90 testes and 90 accessory glands) were taken at 5 X magnification. Testes area and accessory gland area was measured using Image J software and procedure as standardized and given before. All the measurements were averaged out over three independently repeated measurements, to account for any measurement error.

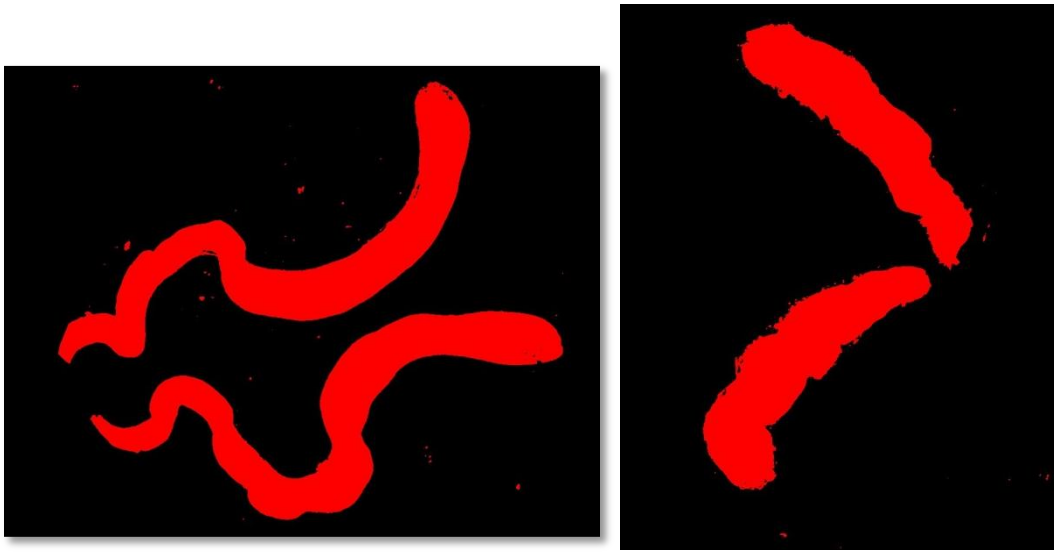


Fig 3.1.3: Measurements of testes area (a) and accessory gland area (b) in image J software by outlining the cross section area (in red color).

3.2 Experiment 2

The study of difference in allometry of reproductive organs over mating, in drosophila melanogaster, selected for different levels of sexual conflict.

Studies have shown the difference in depletion pattern of accessory gland proteins after mating, in lab population of Drosophila melanogaster, selected for different levels of sexual selection (Linklater*et al.* 2007). After the allometry study of reproductive organs of M and F population as virgin, it would be worthy to see the depletion pattern in these selected lines.

3.2.a Experimental procedure

Like the previous experiment, egg collection was done from the standardized M and F population flies. For each population, 10 vials of eggs with density of 150 eggs per vial, were collected and transferred to 8 drams vials containing 8-10 ml cornmeal molasses food. Similarly, 10 vials of eggs with egg density of 150 eggs per vial was collected from LH-st flies were collected and transferred to 8 dram vials containing 8-10 mL cornmeal molasses food.

On day 9-10, virgin males were collected from M and F population and virgin females were collected from LH-st population. For each M and F population, 20 vials with 8 males per vial were collected and for LH-st, 20 vials with 10 females each, were collected. The flies were kept in the 8 dram vials with 4-5 mL food for 2 days.

On day 12, 10 vials of M males are combined with 10 vials of LH-st females and 10 vials of F males are combined with 10 vials of LH-st females. Female number being higher than male number ensures mating of each male in the vial. All the vials are kept for observation to ensure mating of each male. In this way mated treatment is setup. The other 10 vials from both M and F population are kept as it is for the virgin treatment. In an hour, all the vials with males which have mated from mated treatment and the vials with the virgin treatment are frozen at $-20^{O}C$.

The frozen flies were then dissected to measure the area of testes and accessory glands, normalized by the proxy for body size, the thorax length. For each treatment from each population, 25 flies were were randomly chosen and dissected. The procedure was repeated for all the 3 blocks of M and F populations.

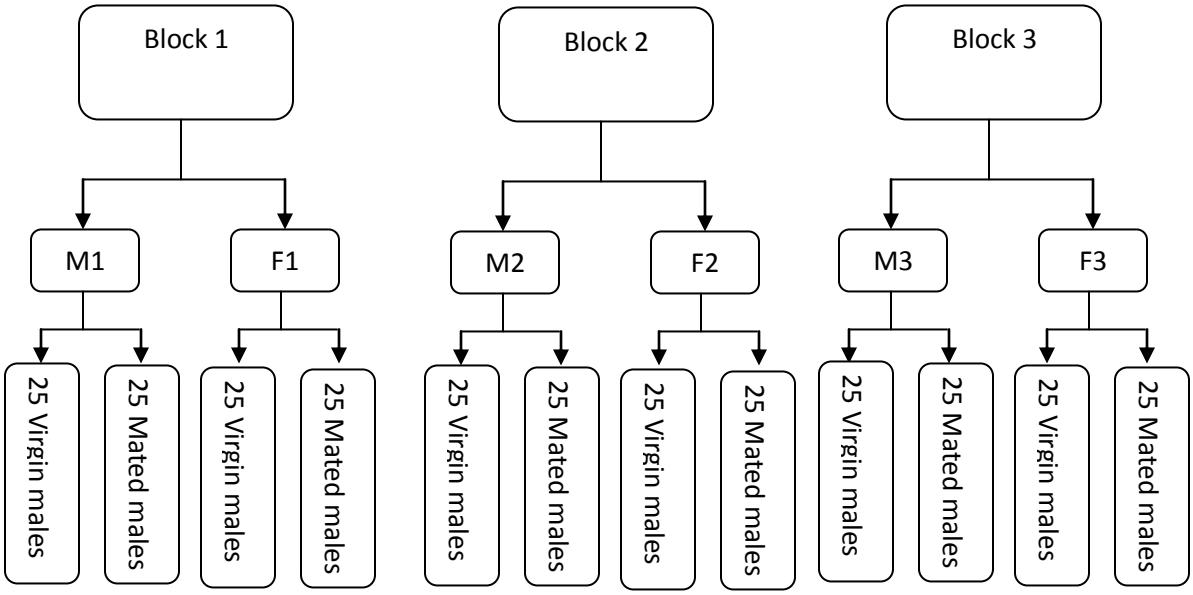


Fig 3.2.1: Experimental setup describing the sample size and mating treatment over three blocks.

3.2.b Thorax length measurement

Thorax length was used as a proxy for body size. All the 300 images were taken at 3.5 X magnification and measurements were done using length tool in Image J software. Each measurement was done three times, independently, to account for any measurement error.

Fig 3.2.2: Thorax length measurement using image J length tool.

3.2.c Testes and Accessory Gland area

All the 600 images (300 testes and 300 accessory glands) were taken at 5 X magnification. Testes area and accessory gland area was measured using Image J software and procedure as standardized and given before. All the measurements were repeated thrice, independently, to account for the error in measurements.

It is important to note that all measurements(i.e., thorax/wing length, testis size and accessory gland size) were taken from the same flies.

RESULTS
CHAPTER 4

Experiment 1

4.1.a Testes area

Actual testes area data was divided by the wing length from the same fly to account for any difference in body size. The values attained were the normalized (Standardized) testes area values.

Mixed model ANOVA with selection regime as fixed and block as random factor was performed on the normalized testes area data.

Testes area of virgin males from M and F populations was used for generating the data and ANOVA was performed. Results revealed no significant difference in testes area across the selection regimes.

Table 4.1: Data-table obtained by performing (ANOVA) using normalized testes size data.

Source	SS	MS	dfnum	df den	F ratio	p value
SELECTION	0.004	0.004	1	2	0.884	0.44
BLOCK&Random	0.002	0.001	2	2	0.259	0.79
SELECTION*BLOCK&Random	0.009	0.004	2	84	4.931	0.009

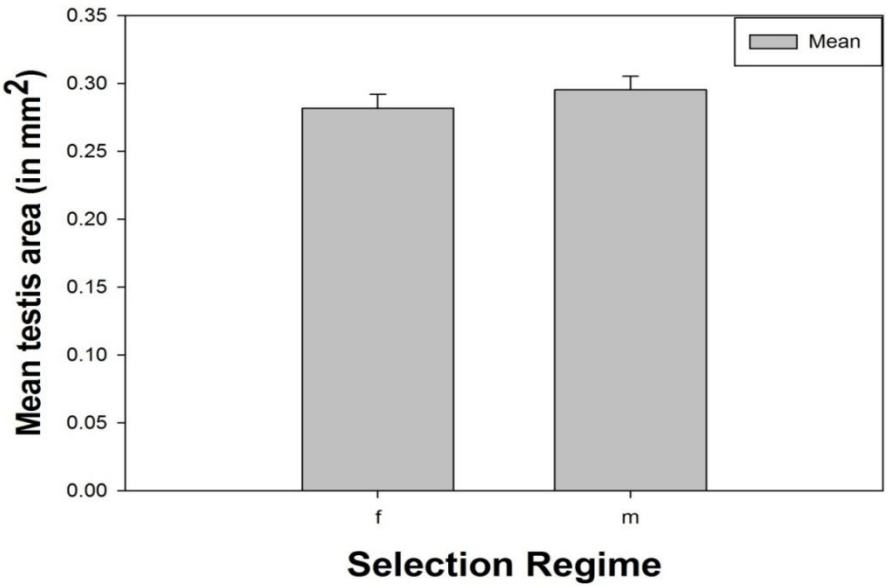


Fig 4.1: Mean testes area (y-axis) and selection regime M and F population (x-axis).

4.1.b Accessory Gland area

Actual accessory gland area data was divided by the wing length from the same fly to account for any difference in body size. The values attained were the normalized (Standardized) testes area values.

Mixed model ANOVA with selection regime as fixed and block as random factor was performed on the normalized accessory gland area data.

Accessory gland area of virgin males from M and F populations was used for generating the data and ANOVA was performed. Results revealed no significant difference in accessory gland area across the selection regimes.

Table 4.2: Data table obtained by performing ANOVA using normalized accessory gland area.

Source	SS	MS	dfnum	df den	F ratio	p value
SELECTION	0.001	0.001	1	1	1.033	0.49
BLOCK&Random	0.006	0.006	1	1	3.464	0.31
SELECTION*BLOCK&Random	0.001	0.001	1	56	4.177	0.04

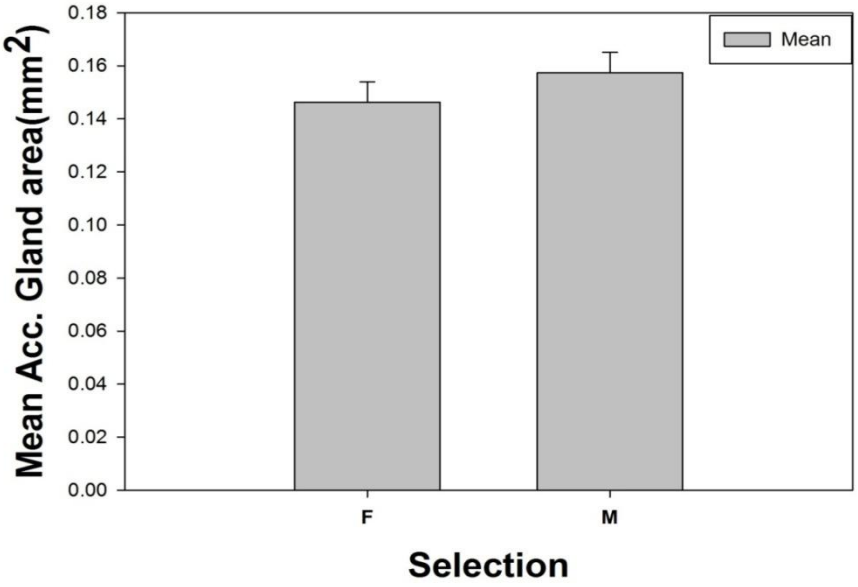


Fig 4.2: Normalized accessory gland area (y-axis) and selection regimes, M and F population (x-axis)

Experiement 2

4.2.a Testes area

Multivariable mixed model ANOVA analysis was performed on the testes area data, using selection regimes and mating status as fixed factor and block as random factor.

Testes area of virgin and mated treatments in males from M and F populations was used for generating the data and multivariable mixed model ANOVA was performed. Results revealed no significant difference in testes area across the selection regimes as well as mating status treatment.

Table 4.3: Data obtained from multivariable mixed model analysis of variance (ANOVA) using normalized testes size data over two treatments.

Source	**dfnum**	**Ddf den**	**f-ratio**	**p-value**
Sel	1	2	0.02	0.907
Mating Status	1	2	0.06	0.829
Block	2	3.5	0.59	0.6
Sel*Mating Status	1	2	2.32	0.267
Sel*Block	2	2	25.68	0.037
Mating Status*Block	2	2	52.53	0.019
Sel*Mating Status*Block	2	287	0.38	0.683

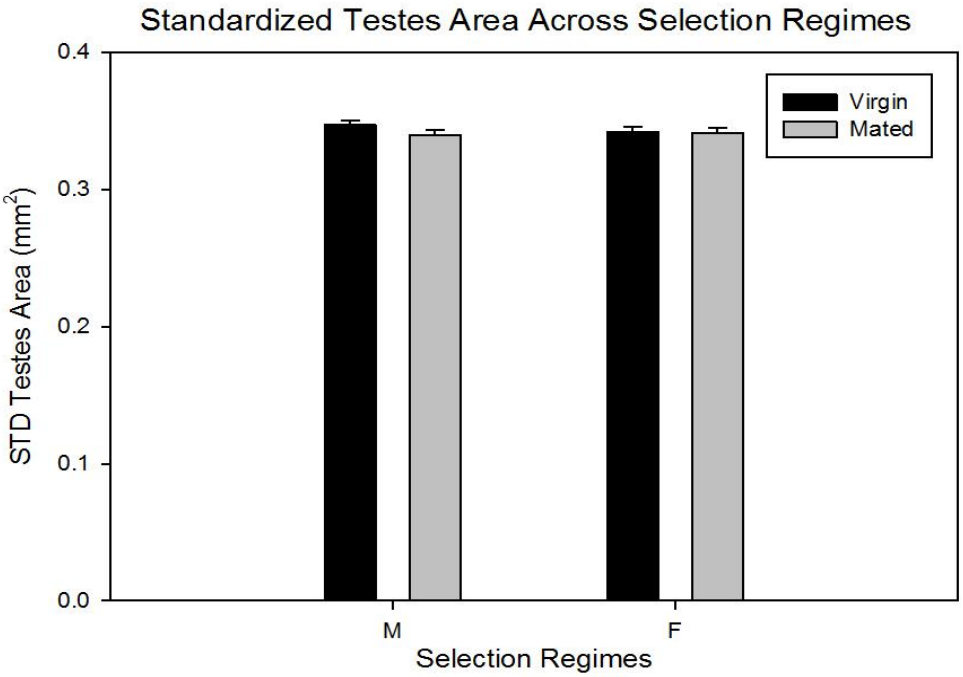


Fig 4.3: Two way interaction graph for normalized testes area (y-axis), selection regimes and treatments (x-axis)

4.2.b Accessory gland area

Mixed model ANOVA analysis was performed on the testes area data, using selection regimes and mating status as fixed factor and block as random factor.

Accessory gland area of virgin and mated treatments in males from M and F populations was used for generating the data and multivariable mixed model ANOVA was performed. Results revealed no significant difference in accessory gland area across the selection regimes. There is a significant effect of treatment. Mated males have significantly smaller accessory glands as compared to virgin males in both selection regimes. But this difference is not significant across the selection regimes.

Table 4.4: Data obtained from multivariable mixed model analysis of variance (ANOVA) using normalized testes size data over two treatments.

Soruce	dfnum	df den	f-ratio	p-value
Sel	1	2	0.3	0.639
Mating Status	1	2	25.05	0.038
Block	2	2.5	5.51	0.123
Sel*Mating Status	1	2	0	0.99
Sel*Block	2	2	2.25	0.308
Mating Status*Block	2	2	7.29	0.121
Sel*Mating Status*Block	2	287	1.42	0.244

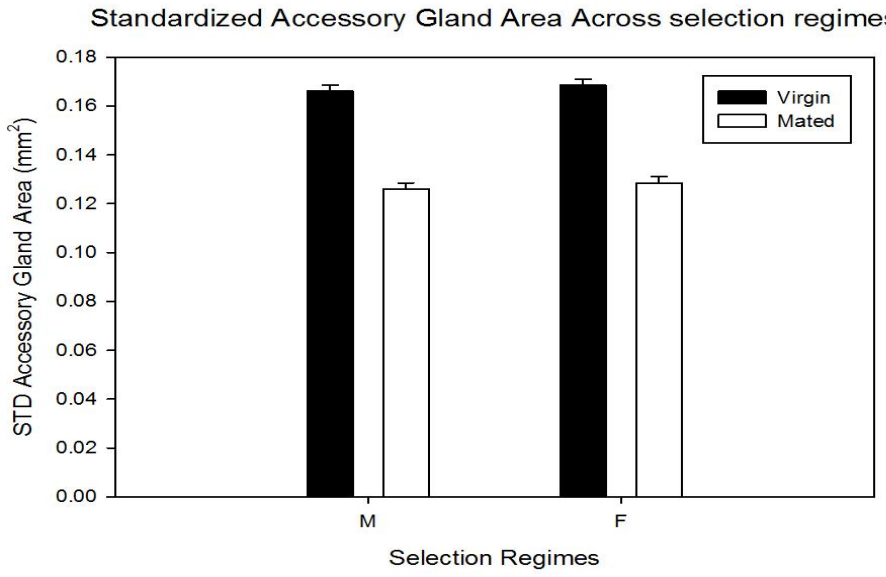


Fig 4.4: Two way interaction graph for normalized accessory gland area (y-axis), selection regimes and treatments (x-axis)

DISCUSSION
Chapter 5

When the populations are maintained in different levels of sexual selection, reproductive investment is predicted to vary for males in population with high sexual selection to ensure they sire higher proportion of progeny. In this study, we did not find any difference in testes size and accessory gland size in populations selected for different levels of sexual selection. The depletion pattern of sperms and ACPs also did not change over a single mating, across the selection regimes. This suggests that there is no difference in reproductive investment across the populations as far as ejaculate quantity is concerned. Different levels of sperm competition and sexual conflict did not lead to evolved testes size. Even the investment in ACPs did not evolve in different levels of sperm competition.

The results show that normalized testes area in virgin males across M and F population is not significantly different. Thus showing that males in M and F population have same reproductive investment and different levels of sexual conflict and sperm competition has not lead to evolved testes size. This result is contradictory to some of the previous studies which suggest that testes size evolve under different levels of sexual selection (Møller *et al.* 1997; Ward et al. 2001) but is in consistence with the previous studies conducted in similar selection lines. (Chapman *et al.* 2004; Linklater*et al.* 2007). We did not find any difference in normalized testes size of M and F population males, after single mating, suggesting that the sperm depletion pattern has not evolved in different levels of sperm competition like in the previous study on similar lines. (Chapman *et al.* 2004; Linklater*et al.* 2007).

We did not find any difference in normalized accessory gland area of virgin males, across M and F populations. This suggests that the investment in ACPs is same by males coming from different levels of sperm competition. Linklater *et al.* 2007 show difference in ACPs depletion pattern in terms of change in accessory gland area after 5 consecutive matings with virgin females, in populations with different levels of sexual selections. However,in this study we did not find any difference in ACPs depletion pattern after single mating with virgin female, suggesting that the ACPs quantity transferred in single mating are not different

in M and F population. This study being more closer to the real senerio where males from M population would have a very low probability of mating with more than one virgin females,

Males from M and F populations with different levels of sexual selection have evolved in terms of fitness. Males from M population have fitness advantage over F males in term of mating success and sperm competition. Males from M population sire higher proportion of progeny after single mating, suggesting evolved response to sperm competition over F males (Nandy *et al.* 2011). But this study suggests that the reproductive investment (in terms of amount of ACPs transferred during mating) in M and F males is the same. Similarly sperm and ACPs depletion pattern does not vary in M and F males, suggesting that the higher proportion of progeny sired by M males is not the result of increased sperm or ACPs transferred per mating.

If it is not the reproductive investment, in terms of testes and accessory gland size, then the fitness advantage could be the result of the quality of sperm produced. M males could be producing relatively better quality sperms and ACPs as compared to F males. The quality can be in terms of sperm length, sperm mobility, sperm mortility and quantitative variations in specificACPs.

This study establishes that reproductive investment does not necessarily vary in populations with different levels of sexual selection as far as reproductive organ sizes are concerned . Any fitness advantages which M males have over F males, does not trace back to physiological changes in testes and accessory gland areas.

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