Exploring the nature of pleiotropy in fitness components in male *Drosophila melanogaster*

Sharmi Sen

MS10012

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

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

April 2015

Certificate of Examination

This is to certify that the dissertation titled "**Exploring the nature of pleiotropy in fitness components in male *Drosophila melanogaster***" submitted by Ms. Sharmi Sen (Reg. No. MS10012) 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. Manjari Jain Dr. N. G. Prasad (Supervisor)

Dated: April 24th , 2015

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.

Sharmi Sen

(MS10012)

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

Warning to the reader: This might look a tad overdone and redundant at places, so do not venture forth if you are one of those impatient, twitchy kind- cause after all it is my acknowledgment!

It is really sad that I would have to restrict myself in thanking the people who have left an impression in my life within these last five years to a page or two. The journey has been sweetly memorable, and I consider myself exceedingly fortunate to be surrounded by people who have made it so. Nevertheless, I shall try, although I am painfully aware of the fact that I am bound to do injustice to a lot of people.

I am grateful to Professor T. R. Rao and Dr. Manjari Jain for their valuable and insightful comments on the thesis.

I would like to thank Dr. N. G. Prasad - he has been the most important person who has single-handedly changed my perception of life and success both in academia and also in the real world. They say that a teacher affects an eternity; one can never really tell where his influence stops. NGP has been the most kind, thoughtful and wonderful mentor that anybody could hope for. It was his passion for science and research that has motivated me to join the academic circle. When I think about it, whatever little that I have achieved so far in these five years, and whatever within my limited capacity I will be achieving in the future, I shall greatly owe it to him. Apart from doing all the mentoring and teaching, he has also generously introduced me to his awesome and amazing

a. Family - It has been statistically tested and proven that NGP throws dinner parties for his lab at a significantly higher rate than any other faculty at IISER Mohali and (most likely the Indian subcontinent). Thank you Jyothi Ma'am for ushering us so welcomingly into your home and making us a part of your lives.

b. his lab -EBL

"Life in lab" evokes the stereotypical image of scientists fervently trying to mix some chemical concoction and desperately reach some conclusion- and I had started out in IISER bearing this picture in mind-but EBL seemed to be diametrically opposite to what I had imagined. So here, I would like to thank my lab members who have been great mentors, friends and to some extent like family. I am grateful to Dr. Bodhisatta Nandy who had initiated me into the routine of designing and conducting experiments and also drawing conclusions from scientific data. He has also derived the SRS flies on which my entire thesis is based but above all he has taught me that research can be fun and exciting. Next off, I would like to thank Vanika Gupta for being an amazing adviser, helping me out in my experiments and feeding the hungry people in lab. I would also like to thank Vinesh for his help with lab work, being so protective and supportive over the past five years, for being a good friend when I needed one and for christening me and my laptop in the lab (albeit uniquely). I shall always remember you whenever I go to a cake shop! Last off on this list, thank you Karan, Lokesh, Aparajita, Neetika, Komal and Nagender- you have been truly wonderful and I wish you all the very best in life.

Thank you Zeeshan for tolerating my idiosyncrasies (especially this past year), for being understanding and forgiving, for helping me out in every single step with my thesis- from experimental design to data analysis. I am really sorry I forced you into staying up all night in order to check my thesis- it was totally uncalled for, I over-reacted as usual. A big vote of thanks to TJ, Manas and Saudamini for their help and support (both in experimental and non-experimental ways). Our lab has always been dominated by the presence of happy Undergrad students who are always ready to help us all out in our work. Thank you Karan, Prakhar, Martik, Ekta, Megha, Vaibhvi for the much needed help while running experiments.

My time at IISER M would have been dull indeed if I did not have my splendid friends to fall back on. I was lucky to have struck the real kind of friendships from my very first year. Thank you Sukriti and Sayali for being the best of friends throughout, the midnight strolls and the birthday parties will never be forgotten. Thank you Preetha, Arya, Cigole, Sukanya, Akshay, Mahima, Kranti, Gargi, Rahul, Arul, Neeraj, Anubhuti, Harshita, Pratip and Srijit for all the pleasant memories and good cheer!

I was also lucky to have had great field research experience during my undergraduate years. My mentors- Dr. Mewa Singh and Dr. Anindya Sinha have been really encouraging over the past three years and have helped me in realising my true potential.

Finally, I would like to thank a few people back home without whom none of this would have been possible. My parents for letting me pursue my dreams and ambitions and for being always supportive and loving and my sister for always being there for me in more ways than one can imagine. Also, I would like to thank the two clowns in my life- Anugruhita and Subhra for being the oldest, funniest and truest of friends.

Fin

List of figures

List of tables

Notation

AP- Antagonistic Pleiotropy

C-S – Canton- S population

SRS- Sex ratio selection line

M- Male biased regime

C- Control or equal sex ratio regime

F- Female biased regime

ANOVA- Analysis of Variance

Contents

Title **Page no**

Abstract

The theory of antagonistic pleiotropy predicts that a trait which provides a fitness advantage in early life but has a deleterious effect at a later life will be selected as strength of selection decreases over age. So far, this theory has been validated with experimental evidences from studies of life history traits such as reproduction and longevity. Little emphasis has been given on the nature of age dependent pleiotropy in traits evolving as a result of sexual conflict. Here, in this study we hypothesize that a sexually selected trait which confers an early life fitness advantage will show rapid decline with age in populations selected for high levels of sexual conflict than in populations with low or intermediate levels of sexual conflict. The trait that we were interested in was male mate harm - a trait that is already known to influence the fitness of a male. Our aim was to investigate whether this trait which has evolved under varying levels sexual conflict shows antagonistic pleiotropy. We have carried out this study using experimental evolution on replicate populations of *Drosophila melanogaster* that have been selected for altered levels of sexual conflict for more than a hundred and forty generation. Previous studies on the very same populations have shown that young males subjected to increased levels of sexual conflict evolve to become more harming to females than young males subjected to intermediate or lower levels of sexual conflict. However, what happens to this trait in selected males at a later age still remains unknown.

We conducted two separate studies in order to understand how flies from selected populations differ in the resources that they acquire early in life and to investigate the effect of age on mate harming ability in males from selected populations and to answer the question whether mate harming ability shows a negative pleiotropy with age.

Our results indicate that selected populations do not differ significantly in the resources that they acquire in their early life and male mate harming ability does show a negative pleiotropy with age in populations with higher levels of sexual conflict. This is to the best of our knowledge the first indication of antagonistic pleiotropy seen in a trait evolving under sexual conflict.

Chapter 1: Introduction

One of the most fundamental problems in Biology that had puzzled scientists for decades was that of aging or senescence. For several years, the scientific community had to struggle with the question: *why do organisms age and why has aging evolved?* Peter Medawar, in 1952, in his book entitled "*An Unsolved problem in Biology*" attempted to address the problem of what makes organisms deteriorate with age without conferring any apparent or obvious advantage. He speculated -in accordance with the theory of "*selection shadow*" (that he and Haldane had postulated earlier in the 1940's)- that the strength of natural selection is greatest at the onset of reproduction and progressively declines with age (Medawar 1952). Therefore, natural selection would generally favour the spread of beneficial alleles at an earlier stage of an organism's lifespan where they would bestow a greater advantage, and allow only those deleterious alleles to accumulate that are expressed later at life to the point where they would hardly ever have a detrimental fitness effect (Kirkwood et al 1979). His conclusion that ageing is the result of the cumulative expression of deleterious genes at a later age is known as the Mutation Accumulation theory.

G. C. Williams extended Medawar's theory to include the possibility of occurrence of pleiotropic genes that have both positive and negative effects on the organism's fitness at different times (Kirkwood et al 1979). Pleiotropy is defined as a condition in which one gene controls or affects more than one trait. Here, antagonistically pleiotropic genes are those that have opposite effects on an organism’s fitness at different ages. Williams' argument was that since the strength of natural selection is maximum at an earlier age and dwindles subsequently with age; traits which confer an early life fitness advantage and that also outweighs the deleterious effects it has on the organism at a later age will be selected for. This came to be known as the theory of antagonistic pleiotropy (AP) which was first proposed by G. C. Williams in 1957 as an evolutionary explanation for senescence (Williams 1957).

There have been multiple studies in the last six decades that have supported the theory of Antagonistic Pleiotropy. Antagonistic pleiotropy has been illustrated through trade-offs such as that occurring between reproduction and survival during early life versus those at a later stage in life (Charmantier et al. 2006). Other confirmatory evidence for the occurrence of antagonistic pleiotropy have come from studies which observed

negative genetic correlations between life-history characters as well as antagonistic indirect responses to selection (Rose et al 2007). Most of these studies have used laboratory experimental evolution techniques (using *Drosophila melanogaster*) to demonstrate that life history traits such as fecundity and lifespan show antagonistic pleiotropy (Rauser et al 2005, Rose et al 2002). Recently, results from long term studies conducted on natural populations of various vertebrate species are also contributing to the growing body of evidence in support of AP (Charmantier et al 2006, Wenburg et al 2004, Bryant et al 2004, Orrel et al 2002).

It is important to note that all these studies have focused on major life history traits that are under the forces of natural selection. However, traits may also evolve as a result of sexual selection. Sexual selection operates through members of a species having an advantage over others in terms of mating and fertilization success. For males, the progression from acquiring a female to successfully mating with her and siring a maximum number of progeny is extremely complicated and the process itself is lined with multiple conditions that need to be fulfilled in order to ensure optimum fitness. In species where females are promiscuous, these complications come in the form of female mate choice (both before and after copulation) and male-male competition. As a result of this, all male traits that are associated with increased reproductive success get selected for. Males are subjected to strong sexual selection through both female mate-choice and intense male-male competition for access to females. This competition amongst males can potentially result in male specific adaptations including the ability to manipulate their mates – either physically or physiologically. Sexual conflict or sexually antagonistic co-evolution lead to the following – (a) evolution of male specific traits that cause mate-harm (Nandy et al 2013)and (b) evolution of traits that triggers resistance in females (Nandy et al 2014). Since both these traits have an immediate consequence on an organism's fitness, hence they can be considered as fitness components of these organisms.

Since mate-harm is caused by male drosophila while courting the female and/or through seminal fluid proteins, it is a resource intensive trait. Hence its amount at various ages can depend on the amount of resources acquired by a male while developing, since that is when drosophila mostly acquires and stores resources (adult males feed minimally). Therefore it is important not to confound pleiotropic effects

with the amount of acquired resources while studying age dependent variations in trait values.

Here, in this present study I tried to answer the following questions:

a. Whether the pattern of resource acquisition has changed in these selected populations at an early stage of life.
b. Whether fitness components (mate harming ability) of male *Drosophila melanogaster* from the selected populations show antagonistic pleiotropy with age.

The study has been conducted using lab based populations that have evolved under varying levels of sexual conflict (see Experimental System for details). The level of intra-sexual conflict was varied by altering the operational sex ratio within these populations. Three sex ratio regimes were adopted – M, male biased (3:1), C, equal sex ratio and F, female biased (1:3). While male biased regime represents increased male-male competition and intersexual conflict, female biased condition represents the opposite. Equal sex ratio represents the ancestral conditions. Earlier studies on the selected populations have shown that young males evolving under higher levels of sexual conflict (M) show greater mate harming ability compared to young males evolving under intermediate (C) and lower (F) levels of conflict (Nandy et al 2013). The work presented in this thesis mainly aims to find out what happens to this trait at subsequent ages.

Chapter 2: Experimental System

Model Organism

Drosophila melanogaster is a holometabolous dipteran insect which has a life cycle consisting of four distinct stages– egg, larva, pupa and adult. Under usual laboratory conditions , i.e., under 25^{o}C temperature, 60-90% relative humidity, their metamorphosis follows the pattern described in (Figure 1). Eggs usually take around 18 to 24 hours to hatch. The larval stage passes through three sub-stages or instars – first, second and third. Maximal resource acquisition for these flies occurs during the larval stages where they dig into the food (the fly media in case of laboratory cultures) and actively feed upon the available food. After attaining a "critical size" the late third instar larva stops feeding, comes out of the food and becomes stationary on some suitable substrate and forms pupa secreting a chitinous covering. The pupa completes development in about 4-5 days. The adult fly comes out of the pupal shell – a process commonly referred to as 'eclosion'. The entire pre-adult 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 start reproducing.

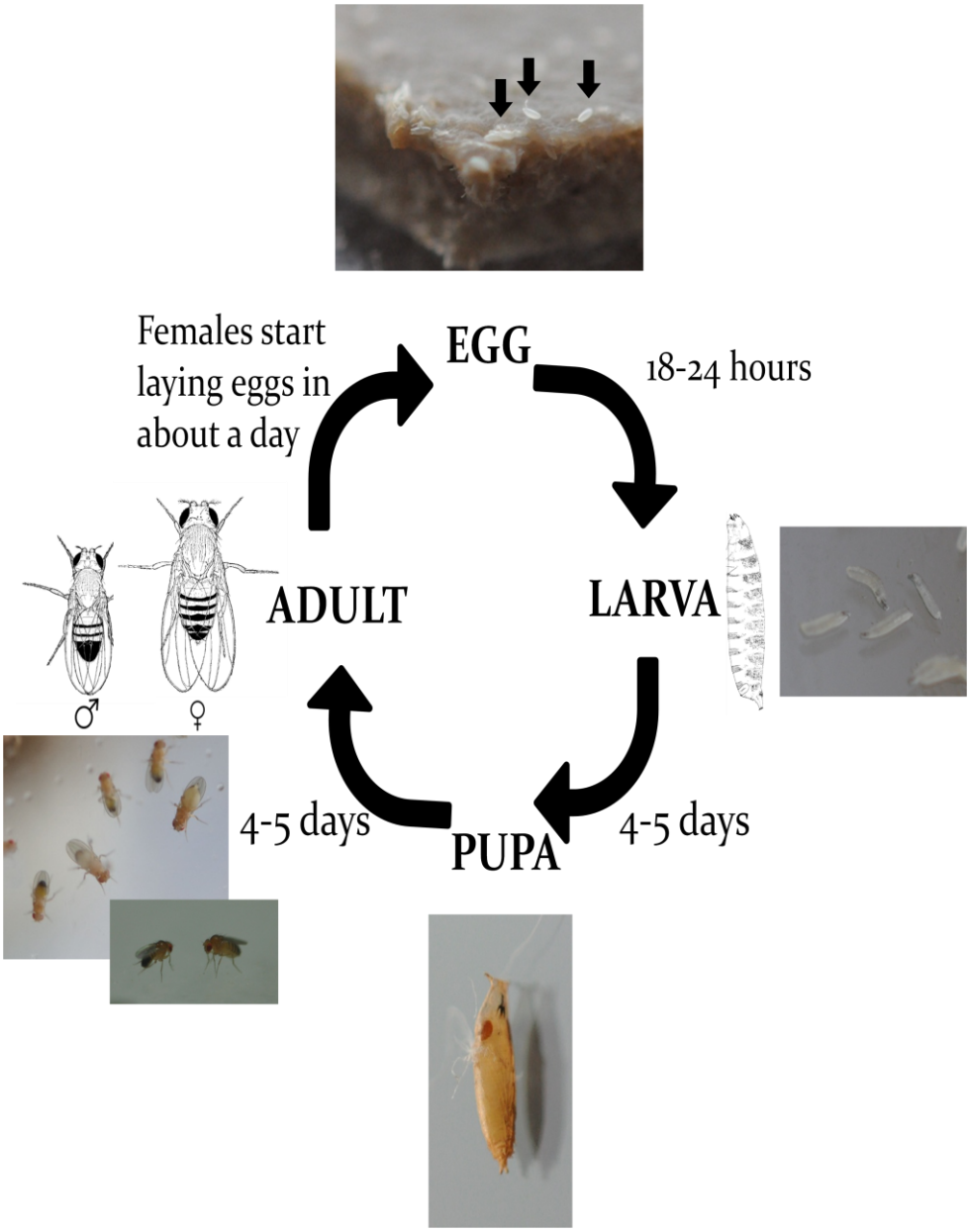

Figure 2.1 - Life cycle of *Drosophila melanogaster*

Laboratory adapted population: LH

Usually a laboratory population is started by collecting a number of mated females from their natural environment and then allowing them to produce progeny in the lab. The offsprings of these wild caught females are then allowed to interbreed amongst themselves. From this generation onward, a large group (here after referred to as a 'population') of interbreeding individuals are maintained under a more or less fixed maintenance regime (temperature, humidity, food, density etc.). These populations are used for experimental purpose only after they have adapted to the laboratory

conditions for several generations. This system allows one to conduct experiments in a manner that avoids inbreeding (by virtue of a large sample size) and also control for environmental variations by making the organisms adapt to laboratory conditions that remain fairly constant for generations. Additionally, these studies ensure that a huge amount of standing genetic variation is preserved within the population by maintaining a large effective size.

There are two populations that have been used for the present study.

a. Sex Ratio Selection Line (also referred as the MCF's)

The MCF's were derived from LH_{st} populations. LH_{st} populations were derived from LH populations (LH-named after the original founder, Larry Harshman). LH population 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 recessive-autosomal trait scarlet-eye ('st') by repeated back crosses (Prasad et al. 2007). Both the populations are maintained under a 14-day discrete generation cycle at 25^{o}C temperature, 60-80% relative humidity and 12-h light / 12-h dark, on standard cornmeal-Yeast-molasses fly food (composition described in the end of this chapter). The populations are kept in 8-dram vials (25mm diameter × 90mm height). A total of 60 vials constitute the LH population whereas the LH_{st} population consists of a total of 30 vials. Every generation, larvae are grown under moderate density (around 150 eggs per 8-10ml of food in 8-dram vials) in 'juvenile competition vials'. On 12th day post egg collection, adult flies across different vials are mixed and distributed (16 males and 16 females per vial) in fresh vials having food seeded with limited amount of live Yeast. This is done under light CO_2-anaesthesia (exposure time < 3 minutes). These vials are referred to as 'adult competition vials'. They are left undisturbed for two days and on 14th day they are transferred to 'oviposition vials' having 8-10ml of fresh food without the use of anaesthesia. They are allowed to oviposit for 18 hours, following which the flies are discarded. The egg densities in these vials are trimmed to around 150 per vial and these now become the juvenile competition vials for the next generation.

The present study was carried out on a set of *D. melanogaster* populations subjected to experimental evolution under different operational sex ratio (ratio of males to

females). The role of interlocus sexual conflict on the evolution of behavioural and life-history traits in this population have already been reported in Nandy et al 2012, 2013. Varying the operational sex ratio within a population results in different levels of male-male competition and interlocus sexual conflict. A male biased sex ratio is expected to create intense competition in between the males, and can generate 'high' conflict condition. Equal sex ratio is the standard ancestral condition. Females biased sex ratio is thought to relax intersexual conflict and male-male competition.

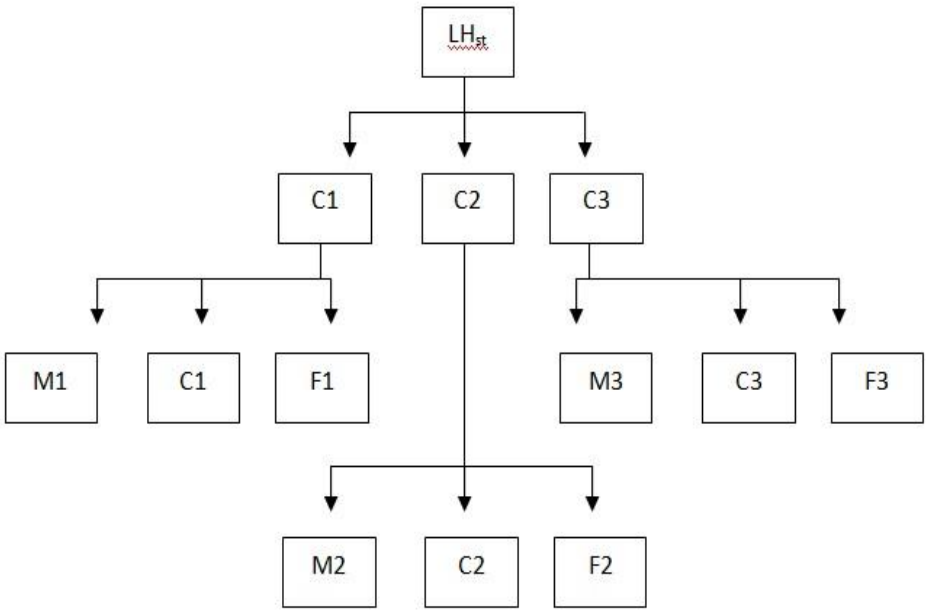

Figure 2.2 - Derivation of MCF- SRS lines from LH_{st}

Three sex ratio regime, each with three replicates were established from the baseline population LH_{st}. Replicates bearing the same numerical subscripts are treated as statistical 'Blocks' in the analysis. All the experiments in this thesis consisted 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 2.3), 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 2.3. 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 9-10 days to complete the pre-adult development. On the 9th and 10th day adult flies start eclosing out of pupal case. The adult flies are collected within 6 hours

after eclosion as virgins and held in single sex vials (pre-reproductive vials). The adult flies take around 8-10 hours to become reproductively mature.

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 (adult competition vials) provided with measured amount of live Yeast. 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.

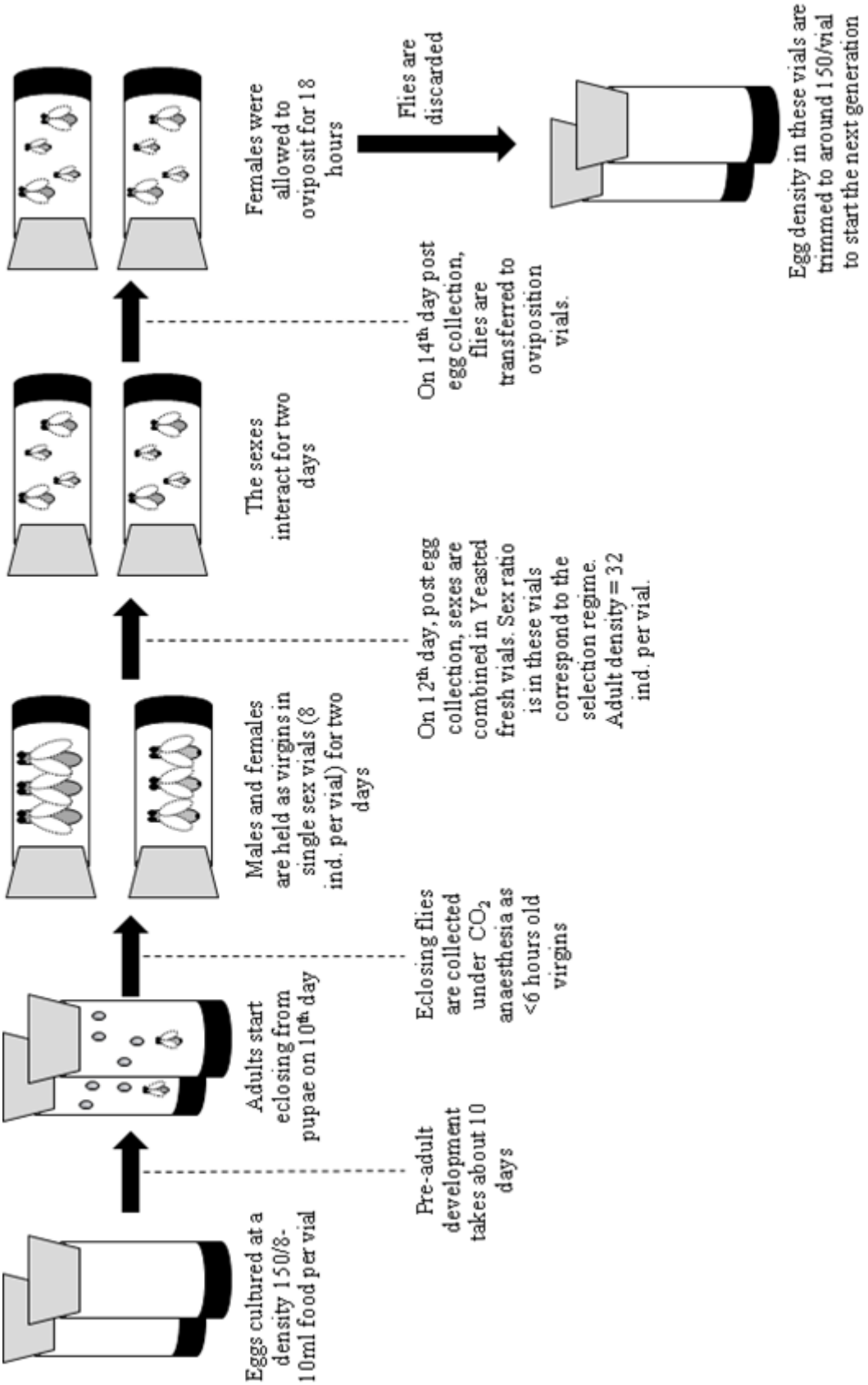

Figure 2.3: Maintenance protocol of selected populations

Ingredient	Amount (per litre of food)
Water (ml)	1100
Agar powder (gm)	14.8
Molasses (ml)	100
Cornmeal (gm)	100
Baker's Yeast (gm)	41.2
Propionic acid (ml)	8
p-Hydroxymethyl benzoate (gm)	2.25
Ethanol (ml)	22.5

Table 2.1- Cornmeal-molasses food recipe for LH lines

b. Canton S

This is a standard inbred population that has been maintained in the lab for several generations. These flies are grown inside bottles containing Banana-Jaggery food (See recipe). Upon eclosion they are sorted into mating pairs of five under light CO_2 anesthesia and transferred into fresh food bottles where they are allowed to lay eggs for 48 hours. The flies eclosing from each of these bottles are again sorted into mating pairs and transferred into fresh bottles. This is done every generation in order to maintain a low effective population size (N_e) and to allow interbreeding between siblings.

Ingredient	Amount (per litre of food)
Water (ml)	1180
Agar powder (gm)	12.4
Banana (gm)	205
Barley flour (gm)	25
Jaggery (unrefined cane sugar) (gm)	35
p-Hydroxymethyl benzoate (gm)	2.4
Ethanol (ml)	45
Baker's Yeast	36

Table 2.2- Banana-Jaggery food recipe for Canton-S

Generation of standardized flies

Even though all other conditions were kept constant across all the regimes, since the three regimes had different environment in terms of their operational sex ratios, it is likely that the immediate environment itself affected the adult condition. Thus it is possible to have some parental effect in the progeny. In the assays described in the following chapters, the aim was to look at evolved genetic differences rather than the immediate parental effect. Hence it was important to equalize the parental effects across different regimes while running the assays. This was done by passing all the populations through one generation of standard maintenance, a process known as standardization (Rose 1984).

During standardization, eggs were collected from the selected populations at the density of 140-160 per 8-10ml of food in each vial. Flies were allowed to grow till adult stage for 12 days under the standard laboratory conditions, 25^{o}C temperature, 60-80% relative humidity, 12 hours light / 12 hours dark. No virgin collection or selection was done. On 12th day post egg collection, flies of each of the populations were transferred to one fly cage (19cm×14cm×24cm) with a petri plate (90mm diameter) having food. The food was smeared with a paste of live Yeast (with water). A cotton piece dipped in water was placed inside the cage in order to prevent desiccation. They were allowed to remain in the cage undisturbed for the following

two days before collecting eggs from them. To collect eggs, they were given a fresh food plate and allowed a window of 18 hours for oviposition. Females lay eggs on the surface of the food. Pieces of food having the desired number of eggs (Usually 150±10) were transferred to food vials having 8-10ml of food. An alternative method of collecting eggs was used while collecting eggs of 'exact' density that is described in the Development time assay.

Chapter 3: Early life resource acquisition patterns

Organisms are often limited by resource availability and or ability of resource acquisition. Therefore with limited resources, an increase in energy allocation in one trait must result in a proportional decrease in resources allocated to another- this is also known as trade-offs between traits. In these experimental populations, the selection pressure dictates that males in M regime should invest more in reproduction than males in F or C regime since they face greater intra-sexual competition. Also, resource availability is the same across the three populations as roughly the same number of larvae feed on roughly the same amount of food and the density is moderate, keeping larval competition at a minimum. If this is the case, then one can expect that the resources acquired by M males in early life will be different than C and F males. In order to investigate whether there is any difference in early life resource acquisition patterns, we checked the i) pre-adult development time ii) dry body weight iii) lipid content of the flies reared under the selection regime described above.

Methods and materials

1. Development time assay

Even though this assay was previously done at a generation number (52-55) and is reported in (Nandy et al 2013) we thought it might be beneficial to re-confirm that result. However, there was one significant difference between the assay that we performed and that described in (Nandy et al 2013) - we used two different densities

a. The same egg density at which the flies are maintained in their selection regime which is 150 egg per vial.
b. A density 60 eggs per vial was also used as in the previous study to check if density has any effect on the development time of these flies.

During standardization the flies from the selected populations were transferred into cages and provided with food and ample amount of live Yeast. Egg collection for this assay was done in a specific manner. Fresh food plate with yeast paste was given thrice consecutively at an

interval of one hour. After the third plate a food plate without any yeast paste was provided and kept for oviposition for three hours. The eggs for the assay were collected from the last plate. This method synchronised the larvae in terms of their

developmental stage. Female *D. Melanogaster* can store eggs inside their body and lay them over a prolonged period of time when a suitable medium is available. This could potentially introduce a lot of asynchrony among the eggs of a single clutch. Therefore the first three plates provided to the females allowed them to get rid of their stored eggs. The last plate was removed after three hours. The eggs from the last plate were transferred to an Agar-gel (1% Agar-agar solution) strip with the help of a fine brush. The eggs were then counted under a microscope on the Agar-gel surface and pieces of Agar-gel with the exact number of eggs required for the two densities (60 and 150) were transferred to 8-dram vials containing 8ml of corn meal food. Time for each of these plate changes was noted down. The mid-point of the time interval for the last plate change and removal of the last plate was taken as h_0 for development time calculation. In order to prevent larva from pupating on cotton plugs, special plugs using muslin cloth and cotton was made. This prevented flies getting crushed between the plug and the vial-wall while taking observation. Vials were given individual identity and kept in the LD incubator under standard laboratory conditions (25 ^{o} C/12:12 LD cycle). The total number of pupae in each vial was also counted to keep a record of the number of eggs that have finished larval development in order to check if there was any difference in larval mortality. Vials were checked for any signs of eclosion from the eighth day onwards. Once the first few flies began to eclose, observations were taken every two- to three hours. Total number of males and females eclosing was noted down at each of these readings. Observations continued for 36-48 hours and ended when the last fly eclosed.

2. Body weight and lipid extraction

In order to quantify how much resource was acquired by these flies in their early life, dry body weight measurements were taken. Flies eclosing from each of the vials were pooled while maintaining their vial identity across different observation points. These flies were then frozen at - 20 ^{o} C. Ten flies of each sex from each vial were collected in micro-centrifuge tubes . The frozen flies were dried at 60^{o}C for 48 hours and weighed in a high precision electronic balance (Sartorius CPA225D) to the nearest 0.01mg. A total of 100 flies of each sex per population per density were measured for body weight distributed in ten groups of 10 each. Mean body weight of each group was calculated and taken as the unit of analysis.

These dried flies were subsequently used for lipid extraction. To each of the aforementioned micro-centrifuge tubes 1.5 mL of diethyl ether was added. The flies were kept in ether for 48 hours on a dancing shaker (gyrator). After this step the excess ether was drained from these micro-centrifuge tubes and the flies were again kept inside the hot air oven for drying for around 24 hours. The body weight of these flies was taken using a high precision electronic balance (Sartorius CPA225D) to the nearest 0.01mg. The difference between the dry body weight and body weight after ether treatment was taken as the amount of lipid. Data from each of the micro-centrifuge tubes was taken as unit of analysis.

Statisical Analysis

For both development time assay and body weight assay a mixed model ANOVA was performed using statistical software JMP (version 9.0) keeping block as a random factor and selection regime, density and sex as fixed factors.

Results

1. Development time

Median development time was calculated as the time at which 50% of the flies had eclosed in each vials. This was done separately for the two sexes. We found a significant effect of selection on the median development across densities and sexes. Selection × Density also had a significant effect. At both the densities, F's develop significantly faster than M's (in both males and females). Males take a longer time to develop compared to females - which is the general case in *Drosophila melanogaster*. There is no significant effect of density on the development time of these flies Density × Selection Regime × Block is also non-significant.

2. Body weight

There was no significant effect of selection or density on the dry body weight of the SRS flies. There was no significant Density × Selection interaction as well.

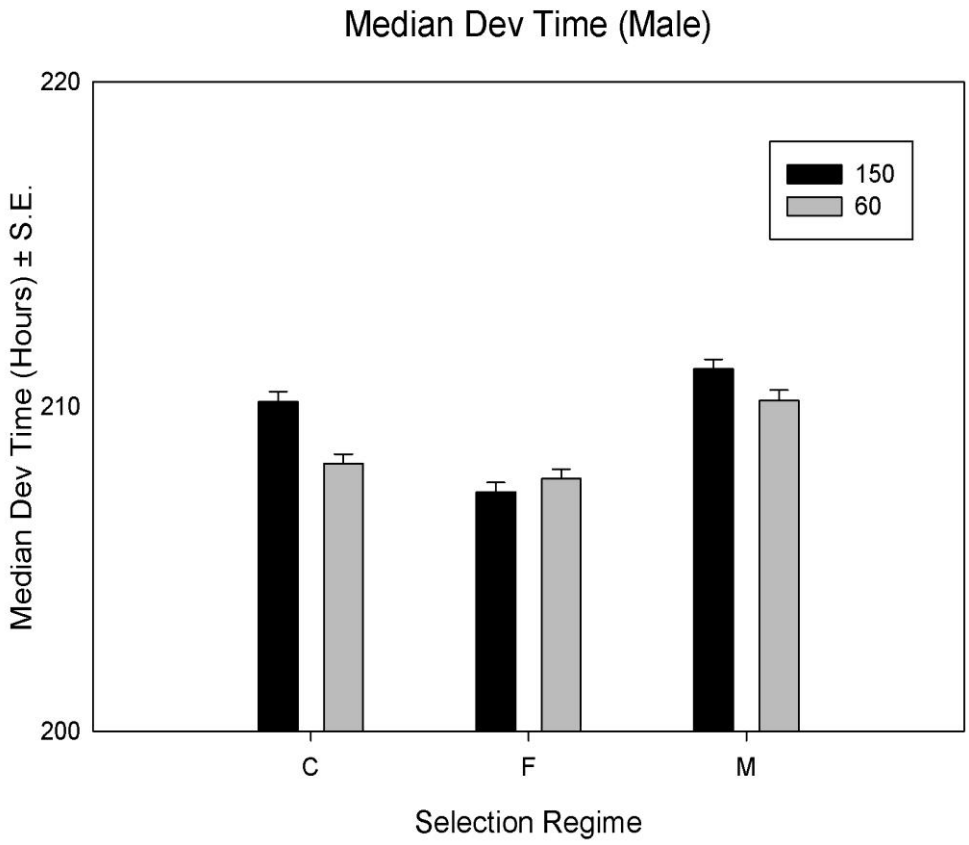

Figure 3.2: Result showing development time of males

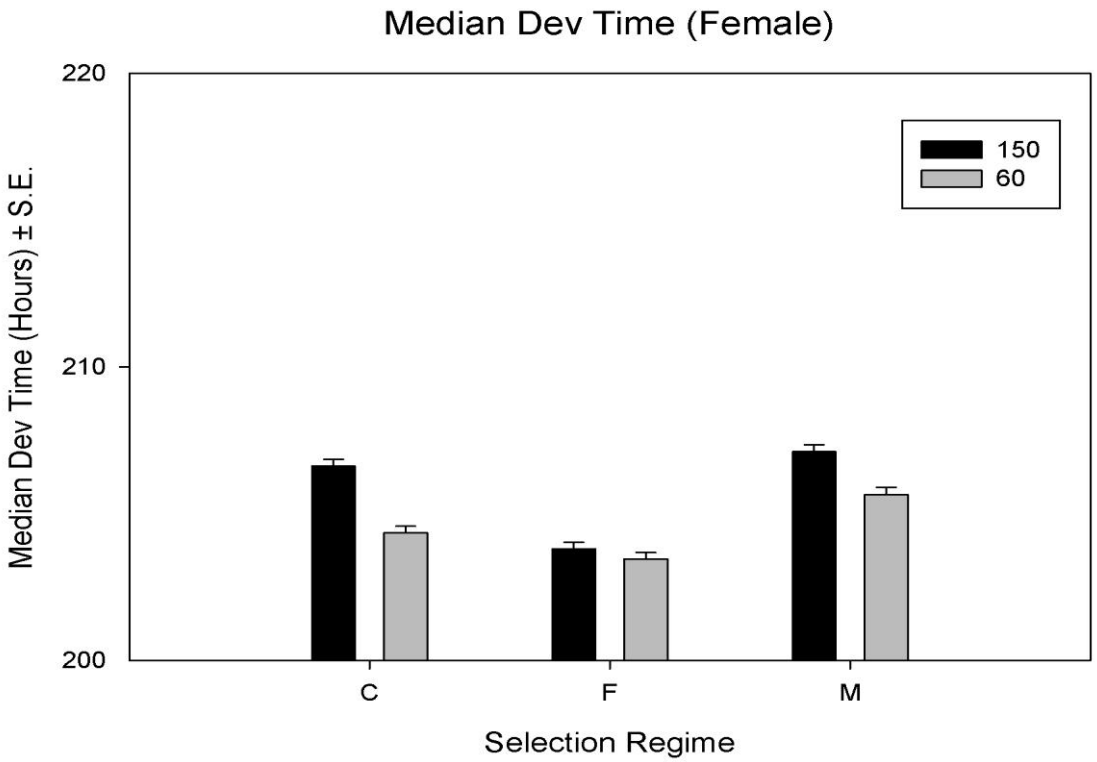

Figure 3.1: Result showing development time of females

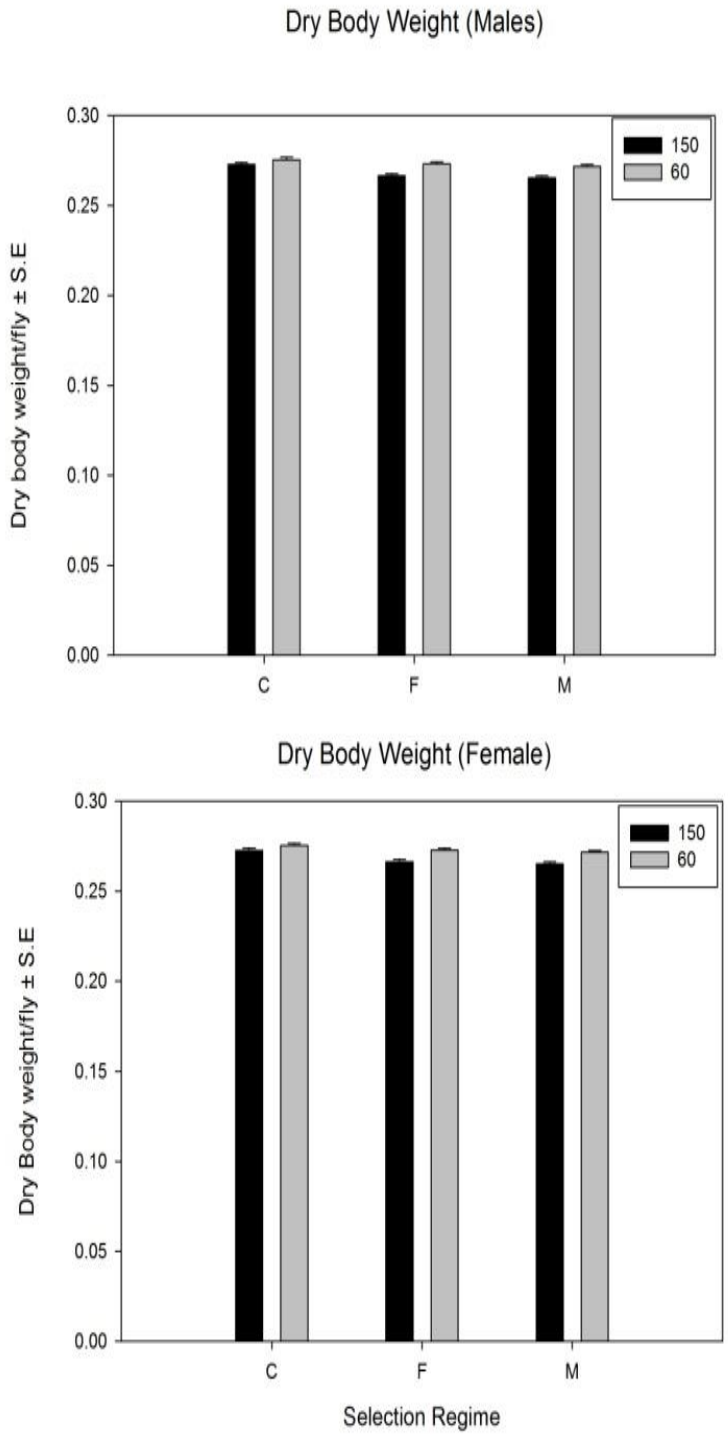

Figure 3.3: Result showing dry body weight of males (top) and females(bottom)

Table 3.1: ANOVA table for pre-adult development time (Males)

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Selection Regime	268.369	134.184	2	15.770	**0.013**
Block&Random	609.466	304.733	2	9.212	0.057
Density	28.136	28.136	1	1.043	0.414
Selection Regime*Block&Random	34.198	8.549	4	3.368	0.133
Selection Regime*Density	39.385	19.692	2	7.776	**0.041**
Block*Density&Random	54.165	27.082	2	10.681	**0.024**
Selection Regime*Block*Density&Random	10.152	2.538	4	1.631	0.169

Table 3.2: ANOVA table for pre-adult development time (Females)

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Selection Regime	224.074	112.037	2	9.674	**0.029**
Block&Random	568.316	284.158	2	7.174	0.062
Density	77.827	77.827	1	2.642	0.245
Selection Regime*Block&Random	46.515	11.628	4	7.532	**0.037**
Selection Regime*Density	27.635	13.817	2	8.940	**0.032**
Block*Density&Random	59.104	29.552	2	19.128	**0.008**
Selection Regime*Block*Density&Random	6.175	1.543	4	0.8127	0.518

Table 3.3: ANOVA table for dry body weight (Males)

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Selection Regime	0.001	0.0002	2	2.379	0.208
Block&Random	0.023	0.012	2	14.92	0.065
Density	0.008	0.007	1	10.229	0.085
Selection Regime*Block&Random	0.001	0.0003	4	0.994	0.501
Selection Regime*Density	0.0016	8.33E-05	2	0.738	0.533
Block*Density&Random	0.002	0.0008	2	6.905	0.050
Selection Regime*Block*Density&Random	0.001	0.0001	4	1.593	0.178

Table 3.4: ANOVA table for dry body weight (Females)

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Selection Regime	0.001	0.001	2	7.368	0.119
Block	0.025	0.012	2	10.694	0.0855
Density&Random	0.001	0.0012	1	1.007	0.417
Selection Regime*Block	0.001	0.0001	4	4.208	0.096
Selection Regime*Density&Random	0.	7.16E-05	2	1.769	0.281
Block*Density&Random	0.002	0.016	2	28.890	0.0041
Selection Regime*Block*Density&Random	0	4.05E-05	4	0.431	0.786

Chapter 4: Male mate harm assay

In polygamous species, males are subjected to strong sexual selection through both female mate-choice and intense male-male competition for access to females. As a result of this, certain male traits that are associated with higher reproductive success get selected for. The competition amongst males to effectively reproduce with a female can potentially result in male specific adaptations including the ability to manipulate their mates – either physically or physiologically. Sexual conflict or sexually antagonistic co-evolution can lead to the following – (a) evolution of male specific traits that cause mate-harm and (b) evolution of traits that trigger resistance in females. Since both these traits are beneficial to the organism's fitness in the way they function and have some consequence on an organism's fitness, they can be considered as fitness components of these organisms.

The manner in which mate-harm is inflicted is fairly species dependent and vary incredibly (Koena 2012, Arnqvist & Rowe 2005). Males may cause mate harm through purely physiological damage (mediated through chemicals transferred along with sperms during mating) or by causing mechanical damage (injuries caused during mating). In *Drosophila melanogaster*, mate harm is caused in two ways :

a. When males court they chase the females around persistently before mating (Fowler & Partridge 1989, Kuijper et al. 2006, Rice et al. 2006).

b. During copulation, they transfer a lot of accessory gland proteins (ACPs) along with sperms into the female reproductive tract (Chapman et al 1995).

These proteins bring about certain physiological changes in the females and make them less eager for subsequent mating with other males. They also suffer fitness costs both in terms of fecundity as well as longevity (Fowler & Partridge 1989, Kuijper et al. 2006, Rice et al. 2006). This is an evolutionary strategy taken up by males in order to ensure that the progeny sired by the female is their own.

It is predicted that when there is higher level of intrasexual conflict, males will evolve greater mate harming ability. A previous study on our experimental system has verified that males from the male biased regime cause more mate harm than males from the Control or Female biased regime. Longevity experiments on males from the

selected populations have shown that M males have lower mean lifespan than C and F males. (Nandy et al 2012)

As already discussed above, traits causing mate harm in females benefit the males and may provide fitness advantages to males at the cost of female survival and fecundity, hence it is an important trait that is under sexual selection. Since the maintenance protocol of these selection regimes dictated that the males and females interact with each other only at a young age (12^{th} to 14^{th} day post egg collection) what happens to this trait at a later age remains largely unknown. Mate harm is quantified by the fecundity and longevity of females housed with males. Lower fecundity and/or longevity indicates higher mate harm.

Here I ask the following question: *Does the ability of males to cause mate-harm decline rapidly with age in populations subjected to higher levels of sexual conflict?*

Materials and methods

Experimental males were generated by collecting eggs from standardised flies (see Standardisation). Eggs were collected on strips of agar plate and transferred into 8 dram cornmeal vials at a density of 150 eggs per vial. Males were collected as virgins and kept in single sex vials containing 8 males per vial. Females used for this assay were taken from the standardised laboratory population Canton-S. Females were also collected as virgins and held in single sex vials for two days. All females used for this assay were young: three day old for the first male age and two day old for the second male age(post eclosion). We assayed mate harm inflicted by males at two different ages. Males were combined on the third day post eclosion with two day old Canton-S females. This treatment will henceforth be referred to Age-1. Fifteen vials were set up for each selection regime (M/C/F) for each block (Block 1/2/3). Observation was not taken on the day of combination and was started two days after combining the males and females. Every alternate day the flies were transferred to fresh food vials and the number of flies alive recorded. Fecundity of the females housed with these males was assayed by transferring the flies into fresh food vials and allowing them to oviposit for three hours after which they were again transferred into fresh food vials. Number of eggs laid on the food vials were the flies were held in the three hour window was counted and noted. Fecundity measurements were done every alternate day on the first week after combining and were gradually spaced apart in the subsequent weeks.

Mortality of flies was checked every alternate day. Whenever a female died, a male was removed from the vial in order to keep the sex ratio equal. Males that were not combined at Age-1 were held in single sex vials and were transferred to fresh food vials every two days. These were used for the next age assay. On the 43rd day post eclosion these males were combined with young C-S females. Ten vials per selection per block were set up. Longevity and fecundity observations were taken from the third day after combining the males and females. Fecundity measurements were done similarly as in Age-1.

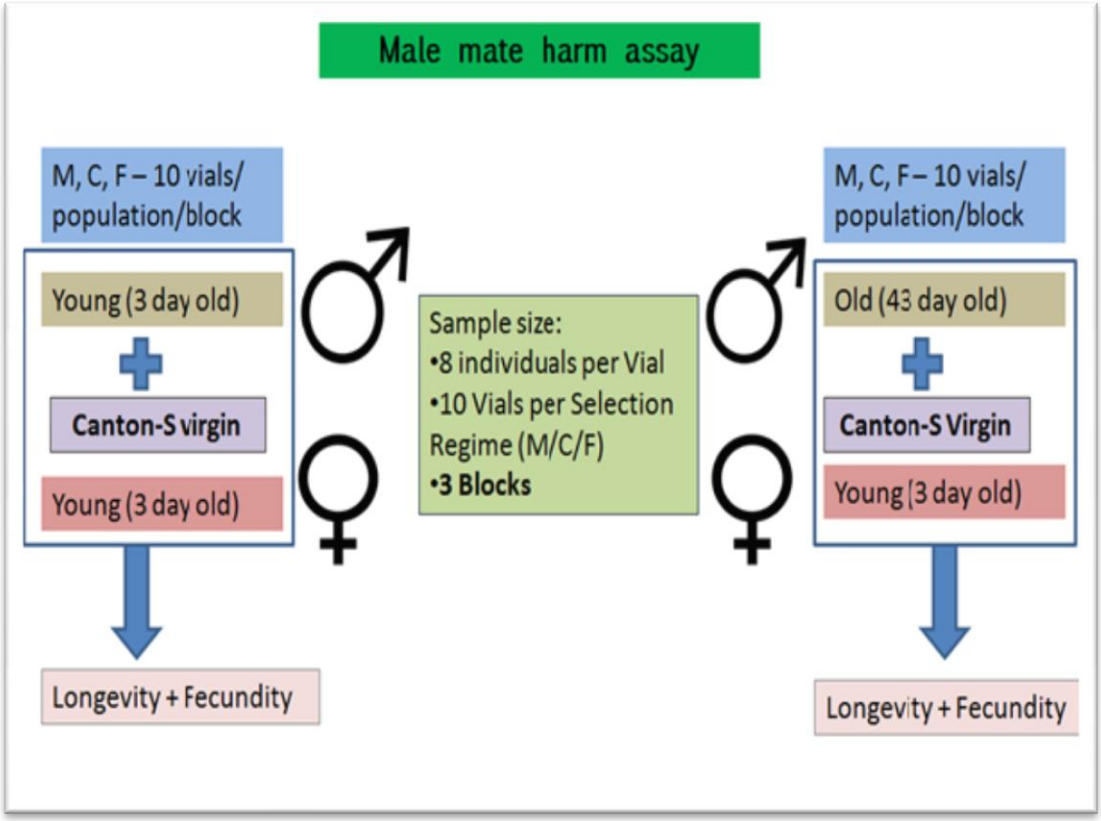

Figure 4.1- Experimental design for mate harm assay

Data analyses

All statistical tests were done using Mixed model ANOVA keeping block as a random factor and treatment as fixed factor in the software JMP (version 9.0).

Results

1. Fecundity:

Number of eggs laid by the female during the three hour window was counted and recorded. We have used the fecundity count of flies for the first eleven days after combining with males for analyses. Analyses revealed that control C-S females when exposed to young M males had the lowest fecundity when compared to females exposed to C and F males. However, C-S females when exposed to old M males had the highest fecundity compared to females housed with old C and F males. Fecundity of M- females at age 1 was significantly different from fecundity of M- females at age 2. In fact, M-females at age 2 had the highest fecundity compared to all the other treatments. This result indicates that M males lose their mate harming ability more rapidly with age than F and C males.

2. Female longevity:

Mortality of females was recorded every alternate day and median time to death was calculated for each selection regime in each block and male age. Analyses revealed that control C-S females when exposed to young males experienced greater mortality and lower longevity when compared to C-S females exposed to old males. Thus, male age had a significant effect on female longevity. However, there was no significant effect of selection regime or selection regime × male age on female longevity.

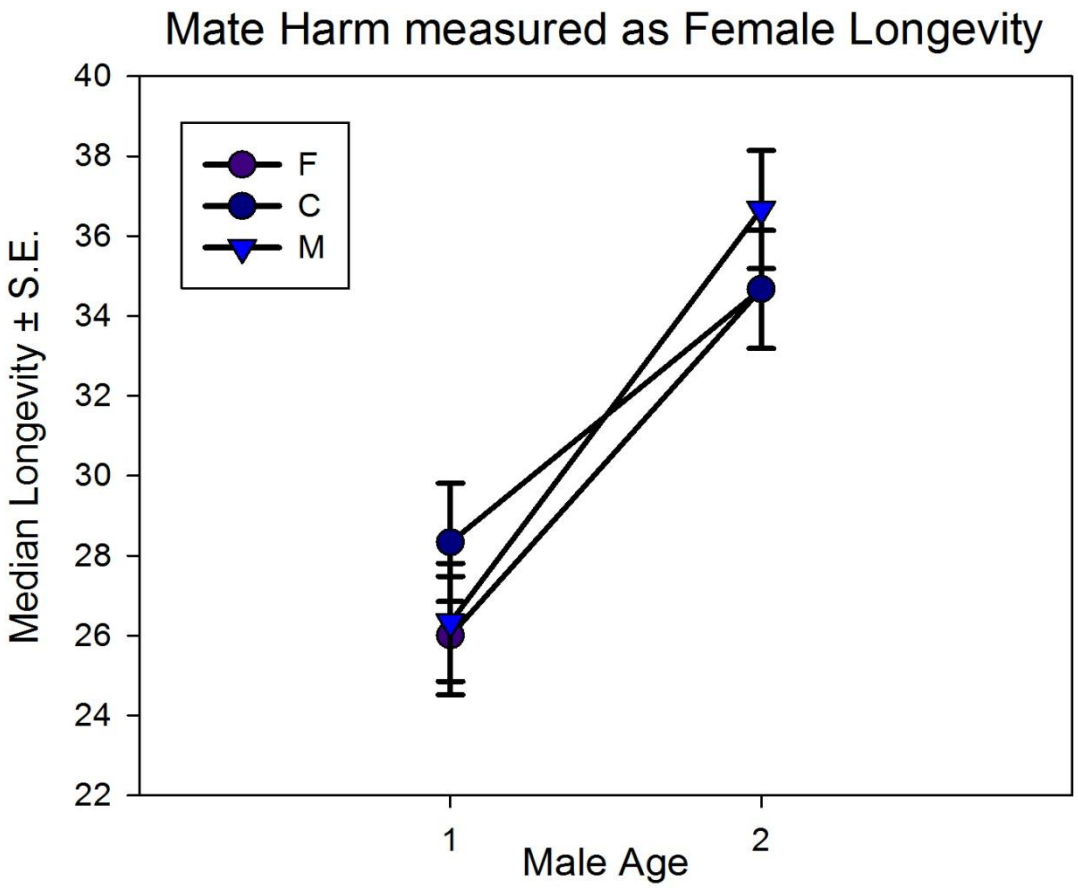

Figure 4.2 - Results showing longevity of females when housed with experimental males from two age classes. Age- 1 being 3 day old males and Age -2 being 43 day old males.

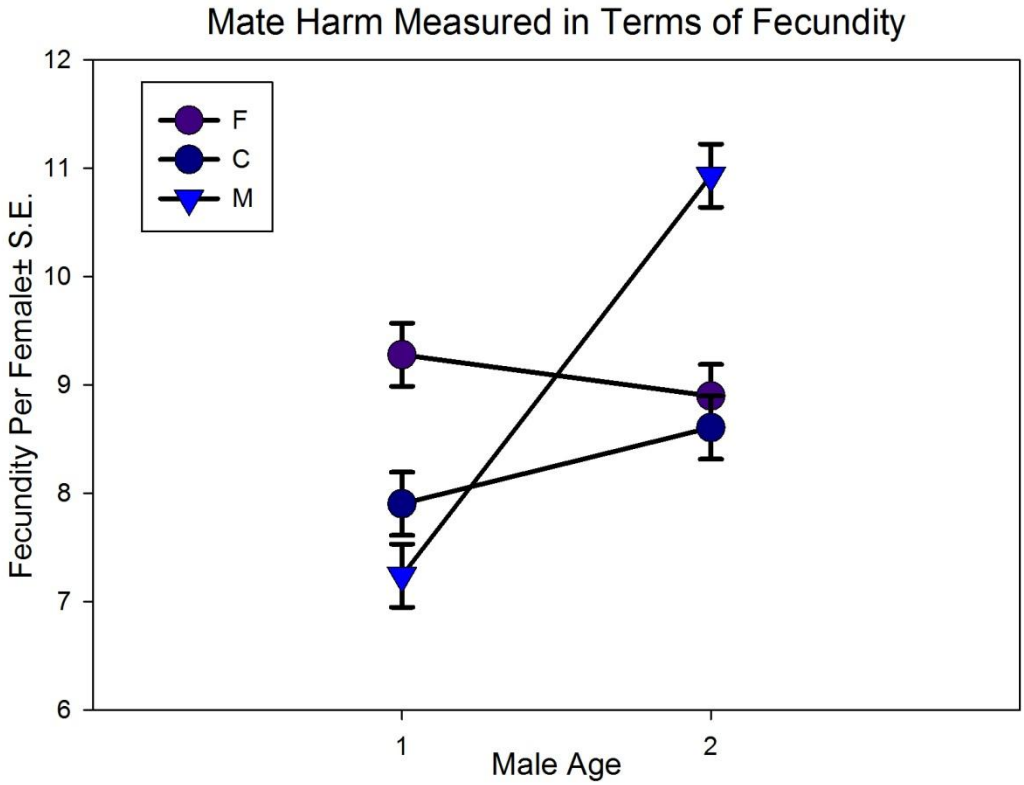

Figure 4.3 - Results showing fecundity of females when housed experimental males from two age classes. Age 1- being 3 day old males and Age -2 being 43 day old males.

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Selection Regime	5.444	2.722	2	1.442	0.338
Block&Random	18.778	9.388	2	-	-
Male age	320.888	320.888	1	94.688	**0.010**
Selection Regime*Block&Random	7.555	1.888	4	0.288	0.872
Selection Regime*Male age	12.111	6.055	2	0.923	0.467
Block*Male age&Random	6.777	3.388	2	0.516	0.631
Selection Regime*Block*Male age&Random	26.222	6.555	4	-	-

Table 4.1 - ANOVA results for female longevity

Source	SS	MS Num	DF Num	F Ratio	Prob > F
Block&random	50.321	25.160	2	0.171	0.853
Selection regime	69.182	34.591	2	3.609	0.127
Male age	201.344	201.344	1	1.399	0.358
Block*selection regime&random	38.337	9.584	4	1.503	0.351
Block*male age&random	287.808	143.904	2	22.580	0.006
Selection regime*male age	333.377	166.689	2	26.155	**0.005**
Block*selection regime*male age&random	25.492	6.373	4	-	-

Table 4.2- ANOVA results for fecundity

Discussion

This present study was carried out in order to investigate whether a fitness related trait that has evolved in response to varying levels of sexual conflict over several generations shows any kind of age dependent pleiotropy. Resources are often limiting and in a situation where there exists intense competition for access to mates, males may

a) Start acquiring more resources early in their life, thereby ensuring that they can preferentially allocate more resources to reproduction in order to optimise their fitness and live for an average amount of time. In this case, since they already have a large amount of resources to begin with, their investment of resources toward reproduction will not rapidly decline with age.

b) Acquire less or the same amount of resources early in their life, rapidly deplete the limited amount of resource that they have gained earlier in life majorly for reproductive purposes, sire a lot of progeny and die sooner. Here, since they do not have an excess of resources to begin with, they allocate more resources towards reproduction at a young age and very little towards the later periods.

In our SRS lines, flies from all the three selection regimes are given the same amount of resources (ad libitum) as they undergo development from egg to adult. Therefore, resources are not limiting in the earliest stages of their life. The selection pressures on these populations are imposed in a way such that the M males are forced to invest more in reproduction (due to intense male-male competition) early in their lives when compared to F males who do not need to invest a lot in reproduction early in life as they are subjected to very low levels of male-male competition. So we wanted to explore if this differential usage of resources for reproduction early in life is manifested through differences in resource acquisition in early life. Previous studies on SRS lines have shown that M males develop faster and have lower mean lifespan compared to C and F males (Nandy et al 2013). I ran the same experiments once again (with important modifications as mentioned in Chapter 2) . Surprisingly, my results show that after more than a hundred generations of selection, the development time of F's have become lower than that of M's in both the sexes- which means that M's take more time to acquire resources than F's. We also measured the dry body weight of these flies which is a good proxy to measure the amount of resources acquired at the onset of their lives. Although M files have a higher development time, the body

weights are not significantly different from that of F's and C's. This could indicate the following:

i) They start out with the same amount of resources in their life.

ii) The resources that they have acquired are differentially used up (not for their somatic growth but maybe for some other function which could be related to reproduction)

Results from the mate harm assay indicates that mate harming ability of M males decline significantly faster with age than both F and C males which is directly reflected on the greater fitness depression of M- females at age 1 and the reverse at age 2. M males have highest mate harming ability when 3 day-old, but that ability is lowest when they are 43 day-old. The longevity analysis shows that young males cause mate harm more than old males. Females when housed with old males live significantly longer than females housed with young males. However, there is no significant difference between the longevity of the females across the three selection regimes at both the ages.

As discussed in Chapter 3, mate harm can have beneficial effects on the male's fitness by increasing the egg production in females and reducing the female's propensity to remate with another male. Males from M regime have evolved to be more harming to the females (as they face intense male-male competition) so that they can ensure the fidelity of the females with whom they mate. In these populations, the opportunity to reproduce is restricted at an early age (as described in the maintenance protocol of these populations). Therefore an antagonistically pleiotropic interaction should result in a rapid decline in the mate harming ability of M males with age compared to the other two regimes.

These results which show a positive correlation between decline in mate harm and early life selection match with this prediction. Thus, it indicates that the decline of mate-harm is due to antagonistic pleiotropy.

Conclusion

In this study, I have tried to find out if a fitness related trait that has evolved in response to varying levels of intra-sexual conflict over several generations shows any kind of age dependent pleiotropy. Preliminary analyses of my data reveal that

a) The males from the three selection regime (M, C and F's) do not differ in the amount of resources that they obtain early in their lives, i.e. while developing from egg to adult.

b) The mate harm incurred by females is the highest when the females are housed with young males from the male biased regime and becomes lowest when they are housed with old males from the same regime. Females housed with young M males at Age 1 have lowest fecundity whereas those housed with old M males at Age 2 have the highest fecundity. Fecundity of females when housed with males from the control and female biased regimes showed no significant variation with age. This indicates that the mate harming ability of M males declines significantly faster with age than compared to C's and F's. To the best of our knowledge, this is the first indication of antagonistic pleiotropy seen in a trait evolving under sexual conflict.

Our results are consistent with our prediction that mate harming ability- a fitness component in males- will rapidly decline with age in populations with higher sexual conflict. However, in order to further substantiate our findings, a thorough examination of the effect of male age on other fitness related traits such as courtship behavior, courtship frequency and sperm competitive ability that have also been earlier reported to have evolved as a response to increased intersexual conflict (Nandy et al 2013) needs to be conducted. Since mate harming ability also leads to the evolution of female specific traits that would render them resistant to the detrimental effects of males, a detailed study of the effect of age on female resistance in the same populations also needs to be carried out. In that case, our hypothesis would be generalized as follows: all traits related to fitness that have evolved in both sexes in M regime will rapidly decline with age. The experimental evidence gathered from the present and future studies will enable us to gain novel insights into the theory of antagonistic pleiotropy and sexual conflict.

References

1. Arnqvist, G. and Rowe, L. 2005. Sexual conflict. Princeton University Press, Princeton, NJ.
2. Bonduriansky, R., A. Maklakov, F. Zajitschek, and R. Brooks. "Sexual selection, sexual conflict and the evolution of ageing and life span." *Functional Ecology* 22, no. 3 (2008): 443-453.
3. Bryant, Michael J., and David Reznick. "Comparative studies of senescence in natural populations of guppies." *The American Naturalist* 163, no. 1 (2004): 55-68.
4. Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F. and Partridge, L. 1995. Cost of mating in Drosophila melanogaster females is mediated by male accessory-gland products. Nature 373:241–244
5. Chapman, Tracey, Göran Arnqvist, Jenny Bangham, and Locke Rowe. "Sexual conflict." *Trends in Ecology & Evolution* 18, no. 1 (2003): 41-47.
6. Chapman, Tracey. "Seminal fluid-mediated fitness traits in Drosophila." *Heredity* 87, no. 5 (2001): 511-521.
7. Charmantier, Anne, Christopher Perrins, Robin H. McCleery, and Ben C. Sheldon. "Age-dependent genetic variance in a life-history trait in the mute swan." *Proceedings of the Royal Society B: Biological Sciences* 273, no. 1583 (2006): 225-232.
8. Chippindale, Adam K., Jonathan R. Gibson, and William R. Rice. "Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in Drosophila." *Proceedings of the National Academy of Sciences* 98, no. 4 (2001): 1671-1675.
9. Fowler, K. and Partridge, L. 1989. A cost of mating in female fruitflies. Nature. 338: 760-761.

10. Fowler, K. and Partridge, L. 1989. A cost of mating in female fruitflies. Nature. 338: 760-761.
11. Jiang, Pan-Pan, Stephanie Bedhomme, N. G. Prasad, and Adam Chippindale. "Sperm competition and mate harm unresponsive to male-limited selection in drosophila: an evolving genetic architecture under domestication." *Evolution* 65, no. 9 (2011): 2448-2460.
12. Kirkwood, Thomas BL, and Robin Holliday. "The evolution of ageing and longevity." *Proceedings of the Royal Society of London. Series B. Biological Sciences* 205, no. 1161 (1979): 531-546.
13. Koene, J. 2012. Sexual conflict in nonhuman animal. In: 1st Ed. The oxford handbook of sexual conflict in humans, edited by Shackelford, T. K. and Goetz, A. T. Oxford Univ. Press, Oxford, U.K
14. Kuijper, B., Stewart, A. D. and Rice, W. R. 2006. The cost of mating rises nonlinearly with copulation frequency in a laboratory population of *Drosophila melanogaster*. J. Evol. Biol. 19: 1795-1802
15. Medawar, P.B. (1952). 'An unsolved problem of biology' HK Lewis and Co.
16. Nandy, B., V. Gupta, N. Udaykumar, M. A. Samant, S. Sen, and N. G. Prasad. "Experimental evolution of female traits under different levels of intersexual conflict in Drosophila melanogaster." *Evolution* 68, no. 2 (2014): 412-425.
17. Nandy, Bodhisatta, Pratip Chakraborty, Vanika Gupta, Syed Zeeshan Ali, and Nagaraj Guru Prasad. "Sperm competitive ability evolves in response to experimental alteration of operational sex ratio." *Evolution* 67, no. 7 (2013): 2133-2141.
18. Nandy, Bodhisatta, Vanika Gupta, Sharmi Sen, Niveda Udaykumar, Manas A. Samant, Syed Z. Ali, and Nagaraj G. Prasad. "Evolution of mate-harm, longevity

and behaviour in male fruit flies subjected to different levels of interlocus conflict." *BMC evolutionary biology* 13, no. 1 (2013): 212.

19. Orell, Markku, and Eduardo J. Belda. "Delayed cost of reproduction and senescence in the willow tit Parus montanus." *Journal of Animal Ecology* 71, no. 1 (2002): 55-64.
20. Prasad, N. G., S. Bedhomme, T. Day, and A. K. Chippindale. "An evolutionary cost of separate genders revealed by male-limited evolution." *The American Naturalist* 169, no. 1 (2007): 29-37.
21. Rauser, Casandra L., John J. Tierney, Sabrina M. Gunion, Gabriel M. Covarrubias, Laurence D. Mueller, and Michael R. Rose. "Evolution of late-life fecundity in Drosophila melanogaster." *Journal of evolutionary biology* 19, no. 1 (2006): 289-301.
22. Rice, W. R., Stewart, A. D., Morrow, E. H., Linder, J. E., Orteiza, N. and Byrne, P. 2006. Assessing sexual conflict in the *Drosophila melanogaster* laboratory model system. Phil. Trans. R. Soc. B. 361: 287-299
23. Rose, M. R. 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution. 38(5): 1004-1010
24. Rose, Michael R., Casandra L. Rauser, Gregory Benford, Margarida Matos, and Laurence D. Mueller. "Hamilton's forces of natural selection after forty years." *Evolution* 61, no. 6 (2007): 1265-1276.
25. Rose, Michael R., Mark D. Drapeau, Puya G. Yazdi, Kandarp H. Shah, Diana B. Moise, Rena R. Thakar, Casandra L. Rauser, and Laurence D. Mueller. "Evolution of late-life mortality in Drosophila melanogaster." *Evolution* 56, no. 10 (2002): 1982-1991.

26. Spencer, Christine C., Christine E. Howell, Amber R. Wright, and Daniel EL Promislow. "Testing an ‘aging gene’in long-lived Drosophila strains: increased longevity depends on sex and genetic background." *Aging Cell* 2, no. 2 (2003): 123-130.

27. Van Valen, Leigh. "A new evolutionary law." *Evolutionary theory* 1 (1973): 1-30.

28. Wigby, S. and Chapman, T. 2004. Female resistance to male harm evolves in response to manipulation of sexual conflict. Evolution, 58(5) 1028-1037

29. Wigby, Stuart, and Tracey Chapman. "Female resistance to male harm evolves in response to manipulation of sexual conflict." *Evolution* 58, no. 5 (2004): 1028-1037.

30. Williams, George C. "Pleiotropy, Natural Selection, and the Evolution of Senescence." *Evolution* (1957): 398-411.