STUDY OF RECIPROCAL CROSS DIFFERENCES IN F1 FEMALES OF DROSOPHILA MAURITIANA AND D. SIMULANS
STUDY OF RECIPROCAL CROSS DIFFERENCES IN F1 FEMALES OF DROSOPHILA MAURITIANA AND D. SIMULANS

By
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Study of reciprocal cross differences in F1 females of *Drosophila mauritiana* and *D. simulans*

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ABSTRACT

Introduction of Haldane’s rule in 1922 was instrumental in advancing the study of speciation. Haldane’s rule states “when in the F1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex”. Since then many studies on hybrid male sterility and in-viability have been done in an effort to better understand the process of speciation in males. Yet the study of speciation in hybrid females has been largely ignored. This does not deter from the fact that females or the homogametic sex can also be affected by speciation, albeit as studies have shown at a much slower pace than compared to males. In our study we re-examined the extent of fertility in F1 reciprocal females of hybridization between Drosophila simulans and D. mauritiana species. Hybridization between these species produces fertile females and sterile males. Our goal was to address the following questions: 1. Are F1 hybrid females fully fertile? 2. Are there any maternal effects observed in reciprocal female hybrids? 3. Are there significant differences in ovariole numbers between the reciprocal hybrids? and 4. What is the state of the hybrid ovaries as a function of age? In order to answer these questions we looked at the level of oviposition and egg hatchability as well as differences in ovariole numbers in pure species and F1 females. Furthermore we proceeded with our experiments with the null hypothesis that the reciprocal hybrid females are not significantly different from each other or the pure species females. Our results indicated that the reciprocal hybrid females are not only fully fertile but they also showed heterosis. Furthermore the heterosis observed in the hybrids can be attributed in part to the presence of maternal effects. The reciprocal hybrids also showed differences in ovariole number compared to each other as well as compared to the parental species. We also found that although the ovariole numbers in hybrids decreased with age, the morphology and structure of their ovaries was still better maintained than the pure species over time which can be attributed to heterosis. In conclusion our findings signify the importance of maternal effects as a potentially powerful mechanism for moderating the rates of evolution of speciation in hybrid females.
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TABLE OF CONTENTS

Abstract........................................................................................................... ii

Acknowledgments.......................................................................................... iii

Table of Contents............................................................................................... iv

List of Figures..................................................................................................... vi

List of Tables....................................................................................................... vii

Introduction...................................................................................................... 1
  1.1 Speciation................................................................................................. 1
  1.2 Hybridization........................................................................................... 5
  1.3 Haldane’s Rule......................................................................................... 7
  1.4 Maternal Effect......................................................................................... 11
  1.5 Drosophila Oogenesis............................................................................... 13
  1.6 Thesis Objectives..................................................................................... 15

Materials and Methods................................................................................... 16
  2.1 Drosophila Stock and Fly Maintenance....................................................... 16
  2.2 Drosophila Hybridizations and Backcrosses............................................ 19
    2.2.1 Fertility Estimates-One Time Mating Hybridization Setup.............. 19
  2.3 Oviposition Rate and Egg Hatchability................................................... 22
    2.3.1 Fertility Measurement After One Mating........................................ 22
    2.3.2 Fertility Measurement After Multiple Matings............................... 23
  2.4 Ovariole Number..................................................................................... 25
  2.5 Statistical Analysis.................................................................................. 27

Results.............................................................................................................. 28
  3.1 Fertility Estimate: one time mating......................................................... 28
LIST OF FIGURES

1.1 Graphical representation of ‘Batson-Dobzhansky-Muller’ model......................... 3
3.1 Fertility estimates of females of both parents and hybrids over 10 days............... 30
3.2 Grand averages of eggs laid/hatched over 10 days, after mating only once......... 31
3.3 Grand average of the percentage of eggs hatched over 10 days, after one time mating ........................................................................................................................................... 32
3.4 Fertility estimates of parental and hybrid female crosses over 10 days after multiple matings........................................................................................................................................... 35
3.5 Grand averages of eggs laid/hatched over 10 days, after multiple matings......... 36
3.6 Grand average of the percentage of eggs hatched for each experimental cross over 10 after multiple matings........................................................................................................................................... 37
3.7 Average ovariole numbers of parental species and hybrid females over 5, 10, 15 and 20 day intervals........................................................................................................................................... 42
3.8 Morphologies of ovaries of pure species and reciprocal hybrid females over time... 44

Supplementary Figures................................................................. 57
SF 1 General picture of reproductive system in mature female Drosophila............ 57
SF 2 Scheme of Drosophila germarium..................................................................... 58
SF 3 Range of morphologies of ovaries of pure species and reciprocal hybrid females over time........................................................................................................................................... 62
LIST OF TABLES

2.1 List of *Drosophila* stocks used in the study.................................................17

2.2 Recipe for standard cornmeal molasses medium for *Drosophila*......................18

2.3 Experimental and F1 reciprocal crosses..........................................................21

2.4 Agar and grape medium.......................................................................................24

3.1 Kruskal Wallis one way Analysis of variance for fertility estimate experiments.....38

3.2 Grand averages of ovariole numbers in *D. simulans, D. mauritiana* and F1 reciprocal hybrids females.................................................................................................41

3.3 Kruskal Wallis one way Analysis of variance of female ovariole counts..........43

Supplementary Tables.................................................................................................59

ST 1 Reproductive relationship between four closely related species of *Drosopila melanogaster* complex.................................................................................................59

ST 2 Summary of trial numbers and results for crosses in fertility estimate-one time mating experiment........................................................................................................60

ST 3 Summary of trial numbers and results for crosses in fertility estimate-one time mating experiment........................................................................................................61
INTRODUCTION

1.1 Speciation

Darwin’s publication of *On the Origin of Species* in 1859 was groundbreaking. In his book Darwin provided many examples to support his arguments on evolution and natural selection in species. Yet, surprisingly he mentioned little on how species come to be (Coyne and Orr 2004). A topic which was viewed as the “mystery of mysteries” for many years to come and one which is better known today as speciation (Coyne and Orr 2004). Reasonably, the road to understanding speciation has not been easy. Questions such as, what are species? How do we define them? How do new species arise? are still being explored today. Adding to this dilemma within academia the view on speciation was divided. Many biologists, Darwin included, did not view species as separate biological entities and instead believed that “species are subjective divisions of nature made for human convenience.” (Coyne and Orr, 2004) Introduction of “Biological Species Concept” in the book entitled *Systematics and the Origin of Species, from the Viewpoint of a Zoologist* by Ernst Mayr provided a consensus for biologists on the definition of species:

“Species are groups of interbreeding natural populations that are reproductively isolated from other such groups” (Mayr, 1942; Coyne and Orr, 2004).

A unified view on species allowed for much needed and neglected studies on the mechanisms of speciation to take precedence. During the period of 1930s and 1940s the renewed interest in the problem of speciation and specifically that of hybrid sterility and in-viability observed in post zygotic isolation finally gave rise to such a mechanism. T. Dobzhansky (1937) and H.J. Muller (1940, 1942) along with much less accredited W. Bateson (1909), each independently provided a solution for the incompatibilities observed in hybrids. Their model now conjointly known as Bateson-Dobzhansky-Muller model provided the first genetic basis for speciation. This model explains that speciation occurs as a result of accumulation of a series of mutations that may be neutral or
advantageous in each of their respective species. However, once these mutations come together in hybrids they are not compatible and thus cause the hybrid to be either infertile or in-viable. Further description of this model is provided in Figure 1.1.
Figure 1.1 Graphical representation of ‘Batson-Dobzhansky-Muller’ model on the rise of genetics incompatibilities in hybrids. The ancestral population has a genotype of AABB. Over time the ancestral population is divided into two isolated populations. A new mutation a arises in one population while in the second population allele B becomes mutated to b. Alleles a and b are mutually incompatible. This is not an issue in pure species populations as these two alleles do not come in contact. However when individuals from these populations mate and produce a hybrid, this incompatibility can negatively impact the fitness of the hybrid. The double headed black arrows indicate the divergence process while the double headed green arrow shows the incompatibility. This figure was adopted from Wu and Ting, 2004.
The period of 1980s saw the rise in study of the so called ‘speciation genes’, those genetic loci that cause reproductive isolation in hybrids. The earliest of these studies began in the form of genetic mapping of such loci. In 1986, Coyne and Charlesworth identified the first gene in *Drosophila* believed to be involved in hybrid male sterility. This gene, the X-linked Odysseus site homeobox or OdsH, causes sterility in male hybrids of *D. simulans* and *D. mauritiana* (Coyne and Charlesworth, 1986; Ting et al., 1998). Later on molecular techniques like gene expression and microarray took precedence in speciation studies. With these techniques biologists were able to compare the expression profile of interspecific hybrids to their parents. In recent years studies by a number of researchers (Singh and Kulathinal 2000, Haerty and Singh 2006, Mavarez et al., 2009) have successfully been able to show that sex biased genes and in particular male specific genes evolve at a much faster rate. Thus, giving credence to the reality of species and the mechanisms which isolates them even more.


1.2 Hybridization

The importance of hybridization on the process of speciation has long been debated. Many scientists regard hybridization and formation of hybrid zones of no particular importance. Indeed even Dobzhansky (1940) and Mayr (1942) as developers of biological species concept viewed gene flow through hybrids as ineffective and hybridization as merely a transitional step within the process of forming fully isolated species (Barton, 2001). Yet others have argued that hybrids may contribute to adaptive variation in existing species and ultimately be a source for new recombinant species (Barton, 2001).

Hybridization can best be defined as ‘reproduction between members of genetically distinct populations’ (Barton & Hewitt, 1985). The outcomes of hybridizations are often complicated due to the endogenous or exogenous selection forces acting on them (Burke and Arnold, 2001). Endogenous selection refers to factors independent of the hybrid environment which result in reduction in fitness in certain hybrids. These reductions in fitness can stem from meiotic irregularities or hybrid incompatibilities. Exogenous selections on the other hand are environmentally induced variations in fitness in hybrids (Burke and Arnold, 2001). For this reason perhaps it is best to view hybridizations as continua (Hochkirch, 2013).

Hybridization produces various recombinant genotypes which have never before been subjected to selection. Majority of these new genotypes will be less adaptive than their parents. As such there will be some degree of selection against the hybrids which will in turn manifest as some form of hybrid inferiority, namely that of hybrid sterility or in-viability (Burke and Arnold, 2001). Classical examples of hybrid inferiority can be found in the studies of hybrid males in \emph{D. simulans} clade. In particular the genetics of sterility in F1 males of \emph{D. simulans} and \emph{D. mauritiana} have been extensively studied (Cabot et al, 1994; Davis et al, 1994; Hollocher and Wu, 1996; Perez and Wu, 1995; Tao et al, 2003).

Sometimes however hybridizations result in hybrids that outperform their parents in characteristics such as greater fertility, developmental speed or biomass (Birchler et al,
This phenomenon is known as hybrid superiority or heterosis. Perhaps the best known example of this is the mule. The mule is the product of crossing a female mare to a male donkey. While the mule is sterile, its superior strength and size in comparison to either parent, has made it the ideal working animal for thousands of years (Plumb, 1920). Unlike hybrid inferiority the genetics of heterosis are not so well understood (Burke and Arnold, 2001). Dominance and overdominance models are two contenders for explaining the genetics of heterosis in hybrids, especially in the F1 generation. Dominance model hypothesizes that recessive or partially recessive deleterious alleles accumulate in parents as a result of inbreeding. However in hybrids the effects of recessive deleterious alleles from one parent can become masked by the dominant allele from the other parent (Crow, 1952). Thus, resulting in a spike in the fitness of the F1 hybrid. Overdominance on the other hand posits that the heterozygote interactions of alleles at certain loci in the hybrids can be superior to the homozygote states found in the parents and as such once again result in heterosis in the hybrid (Crow, 1952, Birchler et al, 2010).

In recent years the search for understanding the genetics of heterosis has moved in a new direction, with epigenetics and maternal effects now also being considered as potential causes for heterosis (Groszmann et al, 2013; Vaiserman et al, 2013).
1.3 Haldane’s Rule

In 1922 the evolutionary biologist J. B. S. Haldane put forth a new rule on the nature of post zygotic isolation which he believed would be applicable to a wide range of taxa, with very few exceptions. His original statement was this:

“when in the F1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex” (Haldane, 1922).

Haldane was indeed correct in his assumptions regarding the heterozygous (heterogametic) sex. In his original paper Haldane provides few examples in cases where males are heterogametic (XY system such as in Drosophila and mammals) as well as where the females are heterogametic (ZW system such as in birds and Lepidoptera) (Haldane, 1922). Since then Haldane’s rule has been scientifically scrutinized and as Haldane originally predicted, it has held true with very few exceptions (Wu and Davis, 1993; Laurie, 1997; Orr, 1997).

One of these few exceptions to Haldane’s rule can be found in a cross made between female Drosophila simulans and male Drosophila melanogaster as was first observed by Sturtevant in 1920. This cross produces sterile males and in-viable females.

Furthermore Haldane’s rule refers to organisms with sex chromosomes, and as such any organism that deviates from this, do not always follow Haldane’s rule. As a result certain types of mosquitoes of genus Aedes (Presgraves and Orr, 1998) are known to deviate from this rule.

Lending support to the universality of Haldane’s rule is the fact that it can also be explained at least in part by the Bateson-Dobzhansky-Muller model (Coyne, 1985). Consequently Haldane’s rule has proven to be instrumental in furthering the understanding of biologists on the concept and the mechanism of post zygotic isolation in particular and speciation in general (Kulathinal and Singh, 2008).

Understandably one aspect of Haldane’s rule has captivated biologist since its inception and that is the question of why? Why is Haldane’s rule so consistent even
across different taxa? Over the years several theories have been proposed to explain the phenomenon of Haldane’s rule, however due to its complex nature, there has not been a single theory that can cover all of the different aspects of Haldane’s rule (Turelli and Orr, 2000). Thus the production of a composite theory seemed to be the logical choice.

Below is a quick overview of the three leading theories on Haldane’s rule. As stated, the dominance, “faster-male” and “faster-X” theories each manage to explain some of the genetic aspects for the noted sterility or in-viability in the heterogametic sex. Lastly we move on to the composite theory which takes a more comprehensive view on the issue at hand (Kulathinal and Singh, 2008).

As previously described dominance theory hypothesizes that alleles that cause decreased fitness in hybrids are partially recessive (Turelli and Orr, 1995). As such in an XY system, if these deleterious recessive alleles which cause post zygotic isolation are X linked they will affect males more than females. Since these alleles will become fully expressed in the hemizygous hybrid males but their effects will be partially masked in heterozygote homogametic females (Turelli and Orr, 1995). This model is also able to cover the large X-effect, also known as the second rule of speciation (Kulathinal and Singh, 2008). This covers the idea that X-linked genes are able to disproportionately affect the fitness of hybrids (Orr, 1997).

The second theory for Haldane’s rule, “faster-X” Theory can be considered a byproduct of the large-X effect. This theory states that X-linked genes evolve faster and thus have the ability to have a greater impact on accumulation of incompatibilities in hybrids (Charlesworth et al, 1987).

The “faster-male” Theory discusses the higher rate of sterility in male hybrids, which is thought to be either due to sexual selection acting strongly in males or that spermatogenesis is a highly sensitive process. However, one limitation of this theory is that it is only applicable to XY male taxa (Wu and Davis, 1993; Orr, 1997).

In 2008 Singh and Kulathinal proposed the “hierarchical faster-sex” theory. This theory encompassed the ‘faster evolution of sex and reproductive-related genes/traits in combination with sex-specific variable evolution of fitness modification by such factors
as dominance and faster-male evolution.' And so it is deemed sufficient as an explanation for Haldane’s rule and is applicable to all sexual taxa (Singh and Kulathinal, 2008)

The search for gaining a better understanding on the genetic aspects of Haldane’s rule has fueled many studies on hybrid male sterility and in-viability and has led to many notable discoveries in this regard. Yet over the years the same search for understanding the parameters of Haldane’s rule with regards to the homogametic hybrid’s state of fertility has been largely ignored. This is perhaps because, many may conclude that by omitting a direct reference to the fertility of females (in the XY system), Haldane was indirectly inferring that female fertility is in fact unaffected and that the females retain their complete fitness. This view was challenged in a study of rescued hybrid female fertility from the cross between D. simulans and D. melanogaster (Hollocher et al, 2000). The hybrids from the cross between D. simulans and D. melanogaster are either sterile or in-viable (Lachaise et al, 1986). The hybridization between these two species is generally considered one of the few exceptions to Haldane’s rule. However, Hollocher and colleagues used rescued fertile female hybrids from these crosses to observe how their fertility may be affected over time (Hollocher et al, 2000). Their results showed that the rescued F1 hybrid females are generally fertile although they had lower amount of adult germline which also degenerated prematurely with age. In addition older rescued hybrids also exhibited mutant egg phenotypes in early oogenesis (Hollocher et al., 2000). Furthermore since hybrid males from these crosses had no detectable germline in adult reproductive tissue, even with use of hybrid sterility rescue, Hollocher and colleagues came to the conclusion that the female and male sterility were as a result of different developmental defects (Hollocher et al, 2000). These results argue for the need to take a closer look at hybrid female fertility in the context of Haldane’s rule. This prompted us to take a closer look at fertility of hybrid females in species that do follow Haldane’s rule to see if they are similarly affected. In our study we have taken up this cause by revisiting the state of fertility in between reciprocal F1 hybrid female of D. simulans and D. mauritiana which are considered to be fertile (Lachaise et al, 1986). Outcome of
hybridizations between four closely related species of D. melanogaster subgroup are shown in Supplementary Table 1.
1.4 Maternal Effect

Maternal effects is the phenomenon where in “the phenotype of an individual is determined not only by its own genotype and the environmental conditions it experiences during development, but also by the phenotype or environment of its mother” (Mousseau and Fox, 1998). We now know that maternal effects are the result of interaction of mother’s gene products (maternal RNAs and proteins) in the zygote’s cytoplasm. Initially the maternal gene products produced from the mother’s nuclear genome are deposited in the egg’s cytoplasm during oogenesis. These maternal gene products are ultimately transmitted through the egg’s cytoplasm to the zygote and are essential for the early development in the zygote before its own genotype can be activated (Vaiserman et al, 2013).

Study on maternal effects began in earlier parts of the 20th century. Earliest publications on the subject came in the form of two successive papers (Donzhansky, 1935) and (Dobzhansky and Strutevant, 1935) on the differences found in testis sizes in the reciprocal crosses of D. pseudoobscura. Dobzhansky attributed these differences to two factors: real cytoplasmic inheritance or maternal effects. He defined real cytoplasmic inheritance as the intrinsic properties of cytoplasm independent of its chromosomes, while referring to maternal effects as ‘the properties of the cytoplasm by those chromosomes [the egg] carried before fertilization’ (Dobzhansky, 1935). However, Dobzhansky ultimately discredits the real cytoplasmic inheritance as a causing factor and concludes the observed differences in testes size are due to maternal effects (Dobzhansky, 1935). This finding noted the first real importance of maternal effects in the evolutionary and developmental processes of organisms.

Three years after the studies on maternal effect by Dobzhansky, Walton and Hamond published their findings on the hybrids of the reciprocal crosses between Shire horse and the Shetland pony. In this classical study the authors primarily focused on weight and relative size differences since birth between the hybrids of these crosses. By the end of the study the authors concluded that the differences observed between the reciprocal hybrids were due to the presence of “maternal regulations” as well as
“nutritional effects” (Walton and Hamond, 1938).

Despite these early and influential studies a limited understanding of how maternal effects originated as well as difficulty in distinguishing it from other similar factors such as cytoplasmic inheritance, meant that maternal effects were often viewed as nuisances (Mousseau et al, 2009). Consequently for a long time maternal effects were considered as nothing more than an unwanted source of variation in experimental data needing to be eliminated (Mousseau and Fox, 1998; Falconer et al, 1996; Wade, 1998). As such the study of maternal effects did not gain much momentum until the late 1980s (Mousseau et al, 2009). In 1987 Roach and Wolfe published the first empirical support for “the near ubiquitous role of maternal effects in plants” (Roach and Wolfe, 1987; Mousseau et al, 2009). Then in 1989 Kirkpatrick and Lande mathematically described “the potential for maternal effects to speed up or deter evolutionary response to selection” (Kirkpatrick and Lande, 1989; Mousseau et al, 2009). Publications of these papers at last sparked an interest in the study of maternal effects. Since then the ubiquitousness of maternal effects in various insects, animals and plants, at different life stages have been well established (Mousseau and Fox 1998).

Reciprocal crosses are often used as a means to study maternal effects. This method is applicable for both the XY and ZW systems. The crosses are performed by reversing the species from which the dam and sire are taken (Vaiserman et al, 2013). The progenies from a reciprocal cross are identical in their nuclear genome. As such, any differences observed between them would point to the presence of maternal effects. Moreover, historically reciprocal crosses have also been used for the study of sex-linkage. In the case of reciprocal hybrid males it would be near impossible to distinguish maternal effects from sex-linkage by only observing the differing phenotypes. However, the effects of sex linkage are negligible when studying the differences in F1 reciprocal hybrid females (Fairbairn and Roff, 2006).

Thus, in our present study we have employed the reciprocal cross method to investigate the presence of maternal effects between the F1 reciprocal hybrid females of D. simulans and D. mauritiana.
1.5 *Drosophila* Oogenesis

At first glance *Drosophila* ovaries look like a pair of tear shaped structures connected at the base via lateral oviducts which come together to form a common oviduct. From there the ovaries are connected to a pair of spermatheca, a single sperm receptacle, uterus and ultimately the vagina (Ogienko, 2007). Uterus is where the eggs are fertilized and the spermatheca and sperm receptacle function as sperm-holders with the majority of the sperm being stored in the receptacle itself (Ogienko, 2007). Each pair of ovaries is made up of ovarioles (Spradling, 1993; Ogienko, 2007). Within a mature ovary each ovariole consists of three regions: the terminal filament at its most distal end, followed by the germarium and the vitellarium (Supplementary Fig. 1) (Ogienko, 2007). In *Drosophila* there are inter-species and inter-strain variations for ovariole number. *D. simulans* are reported to have an average of 15-18 ovarioles per ovary while the average ovariole numbers for *D. mauritiana* are generally lower at 13-15 ovarioles per ovary (Orgogozo et al, 2006).

The germarium is where the development of the oocyte first begins. It can be categorized into four regions 1, 2A, 2B and 3. The terminal filament is at the anterior of the germarium (Supplementary Figure 2) (Ogienko, 2007). Region 1 is where oogenesis first initiates. It contains stem cells from which all ovarian germline cells are produced. Here the stem cell divides to give rise to two new cells: A cystoblast and a replacement for the maternal stem cell. In *D. melanogaster*, the cystoblast continues to divide four more times until it forms a 16 cell cluster. What helps orient the division of stem cell and cystoblasts is a small spherical organelle called spectosome. During the first stem cell division the spectosome is also split. The cell with a smaller amount of spectorosome goes on to become a cystoblast while the other remains a stem cell (Ogienko, 2007).

In *Drosophila* the division is accompanied by incomplete cytokinesis. This result in formation of bridges called ring canals connecting all the cells. During these divisions the fusome is able to pass through all these canals and connect all the cells together.
Cytokinesis occurs in a way that among the 16, two cells will have the highest number of ring canals: four. It is during passage through region 2A that one of these cells will finally be chosen as the prospective oocyte. This cell that is also the oldest of the 16 contains the highest amount of fusome and as such it is within this cell where all the protein and organelles required for oocyte’s development will ultimately gather. From this point on the other unselected 15 cells, which are now dubbed nurse cells begin to nourish the young oocyte via a network of microtubules. In between the two secondary regions of the germarium all cysts become surrounded by follicular cells. These cells, which also originate from the aforementioned stem cells, not only surround each cyst but also proceed to completely separate from one another (Ogienko, 2007). In region 2B only the oocyte is able to undergo meiosis, the process of which is arrested in prophase I and according to sources will not continue until stage 10 in egg chamber development (Ogienko, 2007). Finally in region 3 of the germarium the oocyte resides in the posterior part of a single round cyst which will then leave the germarium for the vitelarium and begin the various stages of egg chamber development (Supplementary Figure 2) (Ogienko, 2007).

It is generally accepted that there are 14 morphological stages to the egg chamber development: stage 1 accounting its entrance to vitelarium and stage 14 being when the egg is mature and ready for fertilization (Figure 4 in the Appendix) (Ogienko, 2007). As the egg chambers mature they are continuously pushed downward towards the posterior end of the ovariole. There are usually around seven egg chambers in various stages of development on a single ovariole (Supplementary Figure 1) (Ogienko, 2007). It is important to note that mutation at any of these stages can have detrimental effects on the development of the oocyte (Ogienko, 2007).
1.6 Thesis Objectives

The aim of this thesis is to study the extent of fertility in hybrid females obtained from reciprocal crosses between *D. mauritiana* and *D. simulans*. As well we wish to address the question of presence of maternal effects between the two reciprocal cross females by studying differences in their egg laying, egg hatching and ovariole numbers. We in turn hope that these data will help us gain some insight on the evolutionary rate of speciation in hybrid females. To do so we will address the following questions:

1. Are F1 hybrid females fully fertile?
2. Are there any maternal effects observed in reciprocal female hybrids?
3. Are there significant differences in ovariole numbers between the reciprocal hybrids?
4. What is the state of the hybrid ovaries as a function of age?

Furthermore, we will proceed based on the null hypothesis that the reciprocal hybrid females are not significantly different from each other or the pure species females. Thus any observed differences between the reciprocal hybrids would indicate the presence of maternal effects. Similarly comparison of the results of oviposition rate and egg hatchability between the hybrids and the parents can determine an answer to the much neglected question of what is the estimate of fertility in F1 hybrid females. Finally, we expect that the results of our study will help further our understanding on the rate of evolution of speciation in females in particular and the process of speciation in general.
Materials and Methods

2.1 Drosophila Stock and Fly Maintenance

The two strains of *Drosophila simulans* (14021.0251.169) and *D. mauritiana* (14021.0241.01) used in this study were obtained from the *Drosophila* Species Stock Centre (DSSC) at University of California, San Diego. Both species were raised on a diet of standard cornmeal and molasses medium supplemented with live yeast grains to promote egg laying. These flies were maintained in 35 ml vials with foam plugs at (22-23 °C) temperature. A description of their origin and stock numbers are listed in Table 2.1. The recipe for the fly medium is given in Table 2.2.
Table 2.1 List of *Drosophila* stocks used in the study

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Stock number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. mauritiana</em></td>
<td>Mauritius</td>
<td>14021-0241.01</td>
<td>University of California, DSSC ¹</td>
</tr>
<tr>
<td><em>D. simulans</em></td>
<td>South Africa</td>
<td>14021-0251.169</td>
<td>University of California, DSSC ¹</td>
</tr>
</tbody>
</table>

¹ from *Drosophila* Species Stock Centre at University of California, San Diego
**Table 2.2 Recipe for standard cornmeal molasses medium for *Drosophila***

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornmeal</td>
<td>78g</td>
</tr>
<tr>
<td>Agar</td>
<td>7.8g</td>
</tr>
<tr>
<td>Yeast</td>
<td>15.6g</td>
</tr>
<tr>
<td>Cold water</td>
<td>204ml</td>
</tr>
<tr>
<td>Boiling water</td>
<td>1030ml</td>
</tr>
<tr>
<td>Molasses</td>
<td>54.6ml</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>6ml</td>
</tr>
</tbody>
</table>

Place a large stir bar in a 2L beaker and mix in cornmeal, agar, yeast and cold water. Place the beaker on hotplate, turn the heat to high and set the stir bar to motion. The rotating stir bar prevents the food from settling at the bottom and burning. Next add in 1030 ml of boiled water and let the mixture come to boil. When the food has boiled for 30 seconds add in the molasses. Mix molasses in thoroughly before removing the food from heat. Once the food has reached 60°C, add in the propionic acid and stir until mixed. Lastly pour the food into vials so that they each contain about 1cm of food at the bottom. Keep the food refrigerated until further use.
2.2 *Drosophila* Hybridizations and Backcrosses

Flies for both inter and intra-specific crosses were collected and sexed as pupae from parental species stocks. The sexed female pupae for each species were kept in separate vials. Each day the eclosed virgin females were transferred to new vials and aged for five days prior to crossing. Both fertility estimates experiments had two intra-specific crosses (within *D. simulans* and *D. mauritiana*) and four F1 hybrid backcrosses; the F1 hybrids females used in these backcrosses were obtained from reciprocal matings of *sim.169♀ x mau.01♂* and *mau.01♀ x sim.169♂* respectively. The two reciprocal crosses resulting in F1 hybrids as well as all six experimental crosses are listed in Table 2.3.

In order to obtain enough F1 hybrid females for these backcrosses, both inter-specific crosses consisted of 10-20 five days old virgin females of one species crossed with 10-20 males of the other species in a food vial. These vials were transferred every other day to prevent larval overcrowding. There were at least three replicates of each reciprocal cross at any one time to ensure enough F1 hybrids were obtained. Since hybrid males were sterile, the female progeny from these crosses were deliberately kept with the males as a means to check for the success of each hybridization. This was done by monitoring these vials for up to 10 days. If any larvae were detected during this time the trials using those females were discarded. No larvae in the vials however meant that the hybridization had in fact been a success.

2.2.1 Fertility Estimates-One Time Mating Hybridization Setup

The virgin females used for this experiment were collected and sexed as pupae, as described in the section above. Four days post eclosion the virgin females were lightly anesthetized using CO₂ and placed in individual vials in preparation for the experiment. Non virgin males of each species were also collected from pure species stocks and placed in groups of five males of the same species per vial. On day five, 1 female and 5 males were crossed together without the use of anesthesia. The vials
containing these flies were then observed until either the flies mated or the trial’s time limit was reached (1hr for intra-specific and 5hrs for inter-specific matings). All six types of crosses performed in this experiment are listed in Table 2.3.
Table 2.3 Experimental and F1 reciprocal crosses. All six experimental crosses numbered on the left hand side of the table. Additionally the two reciprocal hybridizations resulting in F1 hybrids, designated by the symbol F1, are also listed. The backcrossed F1 progeny are not listed in the table since the experiments stopped before the hybrids reached adulthood. Abbreviations: *mau, D. mauritiana; sim, D. simulans* followed by the last digits of their specific strain numbers allocated by *Drosophila* Species Stock Centre at University of California, San Diego. In all crosses the female parent is designated first.

<table>
<thead>
<tr>
<th>Female (♀)</th>
<th>Male (♂)</th>
<th>Cross</th>
<th>Progeny Used for Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pure species and F1s</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>sim.169</td>
<td>sim.169</td>
<td>sim♀ x sim♂</td>
</tr>
<tr>
<td>2</td>
<td>mau.01</td>
<td>mau.01</td>
<td>mau♀ x mau♂</td>
</tr>
<tr>
<td>F1</td>
<td>sim.169</td>
<td>mau.01</td>
<td>sim♀ x mau♂</td>
</tr>
<tr>
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<td>mau.01</td>
<td>sim.169</td>
<td>mau♀ x sim♂</td>
</tr>
<tr>
<td><strong>F1 Backcrosses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F1(sm)</td>
<td>sim.169</td>
<td>F1(sm)♀ x sim♂</td>
</tr>
<tr>
<td>4</td>
<td>F1(sm)</td>
<td>mau.01</td>
<td>F1(sm)♀ x mau♂</td>
</tr>
<tr>
<td>5</td>
<td>F1(ms)</td>
<td>sim.169</td>
<td>F1(ms)♀ x sim♂</td>
</tr>
<tr>
<td>6</td>
<td>F1(ms)</td>
<td>mau.01</td>
<td>F1(ms)♀ x mau♂</td>
</tr>
</tbody>
</table>

* The F1 reciprocal female hybrids are distinguished from each other by the first letter of the female parent followed by initial of the male parent inside the parenthesis. The female parent is always listed first. The female symbol given after the parenthesis corresponds to the sex of the F1 hybrid.
2.3 Oviposition Rate and Egg Hatchability

Two sets of experiments were run to measure the fertility of hybrid females as compared with parental females under the same environment. The measurements were based on the amount of egg deposition and egg hatchability in female hybrids when back crossed to *D. simulans* or *D. mauritiana* males in 1:5 ratio. The methods used in these two studies were modified from a similar experiment (Price et al., 2001). For our two experiments the fertility of females were measured for 10 consecutive days. In either case the trials which were not completed due to the female fly being killed or escaping, or in some cases where no eggs were hatched as a result of unsuccessful fertilization, were eliminated from the final analysis.

2.3.1 Fertility Measurement After One Mating

Immediately after copulation (as described in section 2.3.1) the female was separated from the males and placed into a 235ml bottle with foam stopper, without using any anesthesia in the process. To successfully transfer the isolated female without her escaping, these transfers were performed under the light of a desk lamp with the bottle held at an angle. The fly would move up towards the light source and away from the open mouth of the vial while the bottle with a funnel at its mouth was positioned underneath. The female was then gently tapped into the bottle and the cap quickly replaced. Prior to the transfer a plastic spoon filled with grape tinted medium (recipe for making grape tinted medium is described in Table 2.4) and a drop of yeast paste on top to promote egg laying, was also placed inside each bottle. These bottles were maintained at room temperature for the duration of the experiment. Each day the old spoon was replaced with a fresh one and the number of eggs laid on it counted using dissecting microscope (Vista vision Stereozoom). The spoons were kept for an additional 24 hours, until the numbers of hatched eggs were also counted and recorded. Egg hatching measurements were based upon the number of empty egg casings observed 24h after the initial egg laying. A minimum of ten trials were run for each of the six experimental
crosses. Experimental crosses are listed in Table 2.3.

2.3.2 Fertility Measurement After Multiple Matings

This experiment was designed to identify any effects multiple matings may have on the fertility of the females as compared with being mated to only once. On the day of the trial the female and five males were placed without anesthesia directly in the 235ml bottle containing the grape tinted medium filled spoon. Same as before the spoons were switched every 24 hours, for 10 days. Each day the number of eggs laid on the spoon and the number of eggs hatched on the previous day’s spoon were counted and recorded. As before a desk lamp was used to draw the flies away from the opening of the bottle while the spoon was being changed. However, since the flies were awake throughout this process, it was not uncommon for a fly to escape the bottle. As a result throughout the ten days lost male flies were replenished with males from parental stock to maintain the 1:5 ratio. These males were carefully removed from the species stock vial by first tapping the flies down, removing the cap and quickly placing an inverted empty vial on top. Then while still holding the openings together so flies could not escape, the vials were placed under lamp light. This promoted the flies in the bottom vial to start climbing into the empty vial. As soon as a few flies were in the top vial, both vials were quickly separated and capped. This process was repeated multiple times with another empty vial until only the desired number of males remained in one vial. Those males were then transferred to the experiment bottle whose males needed to be replenished using the desk lamp light as described above. If any female escaped before an experiment could be finished however, that trial was discarded.
### Table 2.4 Agar and grape medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar powder</td>
<td>6g</td>
</tr>
<tr>
<td>Kirkland grape juice from concentrate</td>
<td>333ml</td>
</tr>
<tr>
<td>Large stir bar</td>
<td></td>
</tr>
</tbody>
</table>

Place the stir bar at the bottom of a 2 L beaker. Add 6 grams of agar powder with 333ml of Kirkland grape juice in the beaker. Place the beaker on high heat with the stir bar on low and let the mixture come to boil. Once it has boiled for 30 seconds remove the beaker from heat and let the mixture cool for a few minutes before pouring the medium into individual spoons arranged beforehand. When the medium has solidified the spoons are refrigerated in 4 °C until used. The medium is prepared every 2-3 days so as to keep the medium used fresh.

*This amount of medium fills approximately 40 spoons.*
2.4 Ovariole Number

*Drosophila* ovariole number can be affected by factors such as environmental variation, temperature and starvation (Wayne et al, 2006). In order to control for this, low density populations were established. Inter and intra-specific crosses containing 6-10 males and 6-10 virgin females were raised in vials of standard cornmeal medium supplemented with yeast grains, at (21–23 °C). The flies were transferred to fresh vials daily to maintain the low population density. These populations included two intra-specific crosses (within *D. simulans* and *D. mauritiana*) and two inter-specific crosses (*D. simulans* females x *D. mauritiana* males and the reciprocal cross *D. mauritiana* females x *D. simulans* males). To obtain enough females for the experiment there were at least three replicated for each of these population. Female progeny were collected each day and allowed to age for 5, 10, 15 and 20 days before the they were dissected and their ovarioles counted. These females were mated before they were dissected. The pure species females were simply kept with their con-specific males while the hybrid females were collected two days before the experiment using CO₂. Pure species males were also collected on the same day in equal quantity to the females. The day before the experiment these females were mated with the males without any anesthesia. *F1(sm)* females were crossed with *D. simulans* males and *F1(ms)* females were crossed with *D. mauritiana* males.

On the day of experiment the flies were transferred to an empty vial without anesthesia and placed on ice for half an hour to knock them out. Once knocked out the females were placed individually on a silicon coated petri dish with some 1x PBS solution to make the dissection easier. Then using two forceps with one holding the distal end of the thorax and the other pinching the distal end of the abdomen the ovaries are gently pulled out. The dissected ovaries are stored in dissecting wells containing 1x PBS solution until all dissections are finished. A total of 40 ovaries were collected from females of each specified cross. Thus the ovarioles for 10 ovaries were counted on days 5, 10, 15 and 20 respectively. All the dissected ovaries were then fixed with solution of potassium dichromate for ten minutes. The advantage of this fixative was that, in
addition to preserving the ovaries, it added a yellowish tint to them which in turn made counting the ovarioles easier. Lastly to count the ovarioles they were gently teased apart using two mounted syringe needles under 40x magnification with a dissecting microscope (Wild m3c Heerbrugg, Switzerland). The numbers of ovarioles for both pair of ovaries were recorded separately. Only complete ovaries containing both ovaries were used in the measurements. To record the condition of ovaries over time a subset of the dissected ovaries from each line at each interval were photographed using dark field (DF) setting under 2x magnification using Leica M165 FC fluorescence microscope and mounted camera.
2.5 Statistical Analysis

Shapiro-Wilk test was run to see if the data were normally distributed. This analysis revealed that not all data were normal. So we proceeded with Kruskal-Wallis one way Analysis of Variance (AOV) a nonparametric test for one way analysis of variance followed by Dunn’s pair-wise comparisons test to identify significant differences between crosses in each of the three experiments. A parametric test such as ANOVA relies on assumption on normality to compare the differences between the means in a given dataset. As such any deviances from normality in a data set can result in inaccurate estimation of p-value with this test. However, Kruskal-Wallis one way AOV, the nonparametric equivalent test to ANOVA, does not rely on assumptions of normality (Sokal and Rohlf, 1981). Like all nonparametric tests it operates by ranking the data, in each group the smallest value is given the rank of one and moving upward from there. Average ranks are assigned in places where the values are equal. Sum of ranks is calculated for each group and the variance of the ranks among groups is then calculated (Sokal and Rohlf, 1981). While Kruskal-Wallis test is less powerful than a parametric test like ANOVA, analyzing all data, regardless of their normality, with the same test should ensure that the results obtained are accurate. The Dunn’s pair-wise comparison test is used to compares the mean ranks of the different test groups and to identify homogeneous mean ranks. All data analysis was done using Statistix 8.0 software (Analytical Software, Tallahassee, FL, USA).
RESULTS

In this section we have presented our findings for each experiment in detail. As a way to complement the results of the two fertility experiments presented here, the values for each of the graphs showing grand averages of eggs laid, grand average of eggs hatched and the percentages of eggs hatched for all crosses in each of these experiments are also summarized in supplementary tables. The results for fertility estimate-one time mating experiment are given in Supplementary Table 2 and the values for fertility estimate-multiple matings experiment are listed in Supplementary Table 3.

3.1 Fertility Estimate- One Time Mating

Fertility can be divided into two components, one is the ability of the female to produce eggs and the other is for those eggs to successfully result in progeny. Also quantifying the numbers of eggs produced and hatched can be an important factor in estimating fertility. For this reason our measurement of fertility was categorized into two parts: the number of eggs laid by females and the number of eggs hatched over the 10 days.

Figure 3.1 summarizes the fertility estimates for females of the pure species and the reciprocal hybrids backcrossed to either *D. simulans* or *D. mauritana* males after only one mating. In *D. simulans* starting on day 1, oviposition increases rapidly, reaching its peak on day 3 before slowly decreasing afterwards as females age. In contrast *D. mauritiana* have a more gradual oviposition rate with highest number of eggs laid in days 5 and 7. There is a sudden drop in egg laying in day 6 which brought the number of eggs laid closer to numbers seen on day 3. After day 7, egg deposition in *D. mauritiana* gradually decreases, though their numbers in these last three days remain higher than *D. simulans* females at the same age. Yet, on average *D. simulans* still lay a slightly higher, though not significantly different, number of eggs than *D. mauritiana* in 10 days (Fig. 3.2A).

The oviposition of hybrid females generally follows a pattern of gradual increase. There are also day to day fluctuations in egg depositions in hybrids. But these drops do
not seem to last for more than two days before the egg laying increases again. Furthermore unlike the pure species these fluctuations in egg laying do not seem to follow an age specific pattern. That being said this does not mean that the egg laying would not eventually decrease as a result of age. Only that given a 10 days window for study, the hybrids generally appear to be more productive with respect to egg laying than the pure species. A comparison of the grand averages of eggs laid over 10 days of all six experimental crosses also supports this statement (Fig. 3.2A). Kruskal-Wallis one way AOV and Dunn’s pair-wise comparison test revealed that oviposition rate in F1(ms) females hybrids is significantly higher than the parental species (F=15.6; d.f. =5, 55; p<0.0001). Although there were no significant differences found between the two reciprocal hybrids, F1(ms) females still laid more eggs irrespective of which male they were backcrossed to than the F1(sm) females; with F1(ms)♀ x mau.01♂ having laid the most number of eggs at an average of (387.1, S.E.=23.6) eggs in 10 days (Table3.1, Fig.3.2).

As mentioned before egg hatching measurements were based upon the number of empty egg casings observed 24h after the initial egg laying. In pure species with the exception of days 9 and 10, egg hatching follows the same general trend as egg laying (Fig.3.1c). D. simulans continue to perform slightly better, though not significantly different, than D. mauritiana (Fig3.1e, Fig.3.2B, Fig.3.3) F1(sm) females had the highest percentage of eggs hatched regardless of the species they were backcrossed to (Fig. 3.3). Conversely F1(ms)♀ x sim♂ had the lowest percentage of eggs hatched 35.6% (Supplementary Table2) of all the experimental groups over the 10 days (Fig. 3.3). Kruskal-Wallis one way AOV followed by Dunn pair-wise comparison test showed that there are significant difference in percentage of eggs hatched between the two reciprocal crosses, and more specifically between F1(sm)♀ x sim♂ and F1(ms)♀ x sim♂ (F=3.32; d.f.=5,55;p<0.05).
Figure 3.1 Fertility estimates of females of both parents and hybrids over 10 days. The females were mated with males only once. Legend abbreviations: $F1(sm)$, $F1(D. simulans♀ x D. mauritiana♂)$; $F1(ms)$, $F1(D. mauritiana♀ x D. simulans♂)$. In each case parents of the hybrids are listed inside the parenthesis. Female parents are designated first. (a,b) Average number of eggs laid in parents and hybrids. (c,d) Average number of eggs hatched in parents and hybrids. (e,f) Average percentage of eggs hatched in parents and hybrids over 10 days.
Figure 3.2 A) Grand averages of eggs laid over 10 days, after mating only once. Eggs numbers in F1(ms) ♀s were significantly different (P<0.0001) from both parental species
B) Grand averages of eggs hatched over 10 days from a single mating. No significant differences found in numbers of egg hatched in 10 days. To calculate each bar in the graph first the sum of all eggs laid (A), hatched (B) in 10 days by each trial female in one group was taken. Lastly the overall average of all the sums were computed. This process was repeated for all experimental crosses to produce the six values represented here.
Figure 3.3 Grand average of the percentage of eggs hatched for each experimental crosses over 10 days as a result of one mating. The grand average was calculated for each group by dividing sum of eggs laid in 10 days with the sum of eggs hatched during that time for each of the trial females and then taking the overall average of those values. This was then repeated for each experimental cross data. Significant differences for the percentage of eggs hatched (P<0.05) between groups are also shown on the graph (*).
3.2 Fertility Estimate – Multiple Matings

Here we allowed the females to mate multiple times over the 10 days. One feature of repeated copulation is that it allows for oviposition to reach its maximum rate (Bouletreau-Merle et al., 1982). The increase in egg production observed in *D. simulans* from day 3-6 and *D. mauritiana* from day 3-7 are the result of such rematings. In fact in comparison with single mating experiments (Fig.3.1a), these rematings resulted in a longer lasting increase in oviposition in both pure species. However, the egg production in these species does eventually decrease as a component of age (Fig.3.4a). However grand averages for egg laying in one time mating experiment for *D. sim* with 181.5 and *D. mau* with 168.5 eggs was still higher than that of coupled females where *D. sim* and *D. mau* each laid a grand average of 174.5 and 165.1 eggs respectively in 10 days (Fig3.2A, Fig.3.5A, Supplementary Tables 2 and 3). This is not unexpected considering that single females are known to produce more eggs than females in small groups (Bouletreau-Merle et al., 1982). The results of remating in hybrids are visible within the first few days where we can see a rapid increase in egg deposition by the hybrids females, followed by a more gradual though fluctuating increase in the case of *F1(sm)♀*, before slowly decreasing in the last two days (Fig. 3.4b). Comparison of results between the two fertility estimate experiments shows that once again even with the high productivity of ovaries in these hybrids their average overall oviposition is still lower than single-mated females (Fig.3.2A, Fig3.5A, Supplementary Tables 2 and 3). The only exception is seen in *F1(ms)♀* backcrossed to *D. sim♂* which on average laid 12.8 more eggs than the single mated females of the first experiment in 10 days (Supplementary Table 3). Kruskal-Wallis one way AOV followed by Dunn’s pair-wise comparison test also showed significant differences in egg laying between reciprocal cross females as well as between *F1(ms)♀* and the pure species (F=19.5; d.f. =5.76; p<0.0001). Egg hatching in *D. simulans* and *D. mauritiana* once again followed the same trend as the numbers of eggs laid (Fig.3.4c). Fig.3.4d can be somewhat misleading, with a trend of low daily egg hatching for *F1(ms)♀* x sim.169♂ and a high daily egg hatching in *F1(ms)♀* x mau.01♂ in 10 days; with the daily egg hatching for two *F1(sm)♀*crosses falling somewhere in between. This
however is only partially true. The factor that needs to be taken into account is that the number of eggs hatched is only meaningful in conjunction with the actual number of eggs laid by each line. The grand percentage of eggs hatched (Fig. 3.6) of the two $F1(sm)♀$ backcrosses are in fact higher than $F1(ms)♀ \times mau.01♂$, albeit not significantly so. However, the differences in percentage of eggs hatched was found to be significant between $F1(ms)♀ \times sim.169♂$ and the reciprocal hybrid crosses; as well as between $D. simulans$ and $F1(sm)♀$ ( $F=30.4$; d.f. =5,76; p<0.0001). Table 3.1 summarizes the Kruskal-Wallis one way AOV analysis for both fertility estimate experiments.
Figure 3.4 Fertility estimates of females of both parental and hybrid crosses over 10 days. The females were allowed to mate multiple times. Legend abbreviations: F1(sm), F1(D. simulans ♀ x D. mauritiana ♂); F1(ms), F1(D. mauritiana ♀ x D. simulans ♂). In each case parents of the hybrids are listed inside the parenthesis. Female parents are designated first (a,b) Average number of eggs laid in parents and hybrids. (c,d) Average number of eggs hatched in parents and hybrids. (e,f) Average percentage of eggs hatched in parents and hybrids over 10 days.
Figure 3.5 A) Grand averages of eggs laid over 10 days, after multiple matings. Eggs numbers in F1(ms) ♀s were significantly different (P<0.0001) from both parental species and the reciprocal hybrid females B) Grand averages of eggs hatched over 10 days after multiple mating. Relevant statistically significant (P<0.0001) groups are shown (*). To calculate each bar in the graph first the sum of all eggs laid (A) or hatched (B) in 10 days by each trial female in one group was taken. Lastly the overall average of all the sums were computed. This process was repeated for all experimental crosses to produce the six values represented here.
Figure 3.6 Grand average of the percentage of eggs hatched for each experimental cross over 10 days where females were free to mate multiple times. Relevant statistically significant (P<0.0001) groups are shown designated by the symbol (*). Calculated for each group by dividing sum of eggs laid in 10 days with the sum of eggs hatched during that time for each of the trial females and then taking the overall average of those values. This was then repeated for each experimental cross data.
Table 3.1 Kruskal-Wallis one way AOV for fertility estimate experiments with one or multiple matings. The degrees of freedom listed in parenthesis represent first the degrees of freedom between groups followed by degrees of freedom within groups.

<table>
<thead>
<tr>
<th>Source</th>
<th>Fertility estimate: One time mating</th>
<th>Fertility estimate: multiple matings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F ratio</td>
</tr>
<tr>
<td>Egg Laying</td>
<td>(5,55)</td>
<td>15.6</td>
</tr>
<tr>
<td>Egg Hatching</td>
<td>(5,55)</td>
<td>2.52</td>
</tr>
<tr>
<td>% of Eggs Hatched</td>
<td>(5,55)</td>
<td>3.32</td>
</tr>
</tbody>
</table>
3.3 Ovariole Number

Table 3.2 gives the ovariole numbers of pure species as well as the reciprocal F1 females over 5, 10, 15 and 20 day intervals post eclosion. As described in materials and methods, all females were mated at least one day before the dissection of their ovaries. No anesthesia was used to transfer the males and females to one vial for mating. Throughout the experiment, the female hybrids ovariole count was higher than the pure species females. Kruskal-Wallis test also found $F1(ms)♀$ ovariole numbers to be significantly higher than either $D. simulans$ and $D. mauritiana$. Furthermore, on days 10 and 15 significant difference in average ovariole count between $F1(sm)♀$ and the $D. mauritiana$ females were also observed (Table 3.3, Fig. 3.7). Furthermore in each interval no significant differences were observed between average ovariole counts of the F1 reciprocal females. Despite this, a study of the average ovariole numbers of the reciprocal hybrids at each of the four day intervals, also reveals that the ovariole counts in $F1(ms)♀$ are still clearly higher with an average of 14.20(S.E=0.33) than $F1(sm)♀$ (Table 3.2). The average ovariole numbers for both pure species and hybrids females did begin to fluctuate and decrease as they aged. Both $D. simulans$ and $D. mauritiana$ females had their lowest average ovariole counts (10.45, S.E= 0.40) and (9.55, S.E. = 0.51) respectively on day 15 before bouncing back slightly on day 20. With the two hybrids however the average ovariole numbers reached their peak values on day 15 before finally decreasing on day 20.

The pictures (Fig.3.8) taken from ovaries of both parents and hybrids throughout the experiment also confirmed the evidence of heterosis found in hybrids, specifically in $F1(ms)♀$ thus far. Figure 3.8 represents a sample of the typical ovaries morphologies for all four groups during days 5, 10, 15 and 20 post eclosion. A more comprehensive view of the range of ovaries seen for each group during each interval of the experiment is shown in Supplementary Figure 3. These figures concur that starting on day 5 hybrids have phenotypically larger ovaries, indicating a higher ovariole number and faster development time of the female reproductive system in these hybrids (Fig. 3.8, Supplementary Figure 3). The hybrid ovaries continue to be typically larger than the pure
species throughout the experiment. F1 reciprocal ovaries also visibly appear different from one another with the $F1(ms)$ female ovaries being typically larger than the $F1(sm)$ ovaries throughout the experiment. Moreover the ovaries in all four groups were affected by age. By day 20 they appeared visibly smaller in size and had fewer mature eggs. In the case of parental species atrophied ovaries with no eggs in the ovariololes were also observed.
Table 3.2 Grand averages of ovariole numbers in *D. simulans*, *D. mauritiana* and F1 reciprocal hybrids females. The ovarioles were counted at 5 days intervals beginning 5 days post eclosion and continued to 20 days post eclosion. All females were mated at least one day prior to their dissection. In all crosses the females are given first followed by the species of males used for matings. In the case of F1 hybrids they were only backcrossed to the same species of males to which their mothers belonged. Abbreviation: n, number of female ovary pairs counted for each day.

<table>
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<tr>
<th>Crosses</th>
<th>n</th>
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<th>Day10</th>
<th>Day15</th>
<th>Day20</th>
</tr>
</thead>
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<td><em>sim</em>♀ x <em>sim</em>♂</td>
<td>10</td>
<td>10.75 (0.31)</td>
<td>11.00 (0.47)</td>
<td>10.45 (0.40)</td>
<td>10.60 (0.49)</td>
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<tr>
<td><em>mau</em>♀ x <em>mau</em>♂</td>
<td>10</td>
<td>10.40 (0.46)</td>
<td>10.45 (0.32)</td>
<td>9.55 (0.51)</td>
<td>10.10 (0.53)</td>
</tr>
<tr>
<td><em>F1(sm)</em>♀ x <em>sim</em>♂</td>
<td>10</td>
<td>12.20 (0.37)</td>
<td>12.70 (0.37)</td>
<td>12.75 (0.34)</td>
<td>12.25 (0.42)</td>
</tr>
<tr>
<td><em>F1(ms)</em>♀ x <em>mau</em>♂</td>
<td>10</td>
<td>14.20 (0.33)</td>
<td>14.60 (0.16)</td>
<td>14.35 (0.45)</td>
<td>13.10 (0.35)</td>
</tr>
</tbody>
</table>
Figure 3.7 Average ovariole numbers of parental species and hybrid females over 5, 10, 15 and 20 day intervals; initiating with mated females five days post eclosion. The bars were produces by first taking the means of ovariole numbers in first and second ovaries of each female and then calculating the mean of all the females tested in each cross. Significant differences between groups at each interval are indicated by the connection lines above the bars and (*) signs. Legend description: D. *sim.*169, D. *simulans*169; D. *mau.*01, D. *mauritiana*01; F1(sm), F1(D. *simulans*♀ x D. *mauritiana*♂); F1(ms), F1(D. *mauritiana*♀ x D. *simulans*♂). In each case parents of the hybrids are listed inside the parenthesis. Female parents are designated first.
Table 3.3 Kruskal-Wallis one way AOV calculated for ovariole counts of parental species and hybrid females over 5, 10, 15 and 20 day intervals. Degrees of freedom listed in parenthesis represent first the degrees of freedom between groups followed by degrees of freedom within groups.

<table>
<thead>
<tr>
<th>Source</th>
<th>Ovariole Count</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F ratio</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Day5</td>
<td>(3,36)</td>
<td>21.4</td>
<td>0.0000</td>
</tr>
<tr>
<td>Day10</td>
<td>(3,36)</td>
<td>31.8</td>
<td>0.0000</td>
</tr>
<tr>
<td>Day15</td>
<td>(3,36)</td>
<td>28.1</td>
<td>0.0000</td>
</tr>
<tr>
<td>Day20</td>
<td>(3,36)</td>
<td>10.5</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
Figure 3.8 Morphologies of ovaries of pure species and reciprocal hybrid females over time.

Samples of typical ovaries morphologies of *D. simulans.169, D. mauritiana.01, F1(sm) and F1(ms)* females dissected on days 5, 10, 15 and 20. The first set of dissections were done at five days post eclosion. All females were mated one day prior to dissection. All images were taken using dark field (DF) setting under 2x magnification using Leica M165 FC fluorescence microscope and mounted camera. All images are to the same scale bar of 1mm. For view of the range of ovaries morphologies in each group in each interval please refer to Supplementary Figure 3.
DISCUSSION

The quest to better understand the process of speciation and in particular the cause of Haldane’s rule has led to many studies being done on the genetics of sterility in hybrid males across various taxa. Studies have also been done on cases where Haldane’s rule is an exception (Presgraves and Orr, 1998). However, in species obeying Haldane’s rule, there have been to our knowledge very few studies which have looked at the fertility of female hybrids and whether their fitness is also affected due to consistence of Haldane’s rule (Lachaise et al, 1986; Davis et al, 1994; Hollocher et al, 2000; Price et al, 2000). As a result the objective of our study was to re-examine the fertility of F1 reciprocal females in D. simulans and D. mauritiana cross. Our findings not only answered the question of the level of fertility in these F1 females but they also provide new data on evolution of speciation in females.

4.1 Fertility of F1 Reciprocal Hybrid Females

The results obtained from the two fertility estimate experiments were of interest. In both experiments regardless of the number of times being mated, the reciprocal hybrid females deposited more eggs than the parental females. Also with the exception of F1(ms)♀x sim.169♂ the percentage of eggs hatched was equal if not better than the parents. Based on these results we can safely conclude that the F1 hybrid females are fully fertile. Our results are also consistent with previous report on the fertility D. simulans and D. mauritiana female hybrids (Lachaise et al, 1985).

Looking at the two fertility experiments individually one can also address the question of the state of fertility of hybrid females as a function of age. In the first experiment the females were mated with males only once. Therefore, these females possessed a limited amount of sperm. As such the decrease in egg hatching observed in the pure species females and to some extent in the hybrid females was more likely due to the exhaustion of spermatozoa rather than age. In contrast the females in the second experiment were mated with multiple times. Periodic copulation has been shown to provide both simultaneously a sufficient amount of sperm and also a permanent stimulus...
for oogenesis (Bouletreau merle et al, 1982). Thus, the eventual decrease in fertility of parents and female hybrids as observed in the second experiment was mainly due to aging.

However as will see, this explanation only goes so far in terms of justifying the significant decrease seen in egg hatching of $F1(ms)\varphi x sim.169\varphi$ in both fertility experiments. Although we did not investigate the reasons for the significant decrease in egg hatchability in $F1(ms)\varphi x sim.169\varphi$ in our study, we believe that it may have been caused by one of two factors: first majority of eggs laid in $F1(ms)\varphi x sim.169\varphi$ may have been unfertilized. Second the decrease in egg hatching may be due to incompatibilities in the $F1(ms)\varphi$ genetic background with that of $sim.169\varphi$. Needless to say further investigations are needed to ascertain the cause(s) for reduction in fertility of $F1(ms)\varphi x sim.169\varphi$ cross.

Second notable aspect of the results was the differences in egg laying with higher egg deposition of $F1(ms)\varphi$ compared to $F1(sm)\varphi$ and egg hatching including lower eggs hatched in $F1(ms)\varphi x sim.169\varphi$ cross, observed between the two reciprocal crosses. These differences indicated the presence of maternal effects in F1 reciprocal female hybrids. This is the case since the reciprocal F1 female progeny of $D. simulans$ and $D. mauritiana$ cross, have inherited identical X chromosomes as well as identical nuclear genomes. As a result reciprocal F1 females differ only in their mitochondrial DNAs and parent of origin effects, ‘including maternal hormones and maternally derived RNAs deposited in the egg’ during oogenesis, inherited from their respective mothers (Vaiserman et al, 2013). To be more precise the difference observed in egg laying in the two experiments can be fully attributed to maternal effects, since oviposition is a maternal sexual trait. In contrast the differences observed in egg hatching as well as the percentage of eggs hatched between the reciprocal hybrid backcrosses in both fertility experiments can only be partially attributed to maternal effect since potential contribution of the paternal genotypes, especially in the case of $F1(ms)\varphi x sim.169\varphi$ cannot be ignored.
The evidence for maternal effects is also supported by the observable differences seen in reciprocal hybrid ovariole numbers. *F1(ms)* females had a higher average ovariole number than *F1(sm)* females throughout the four intervals for which the ovariole numbers were counted (Table 3.2). Furthermore, in *Drosophila* ovariole number has also been shown to be strongly correlated with egg production (Cohet and David, 1978). Our results for egg laying also supported this. The high ovariole count of *F1(ms)*♀ also correlate with the fertility experiments results. In both fertility experiments the *F1(ms)* females laid more eggs than *F1(sm)* females regardless of the species they were back crossed to, with the oviposition being significantly higher in the second fertility experiment where the females were mated males with multiple times.

### 4.2 F1 Reciprocal Females Display Heterosis

A third notable aspect of the results was the clear display of heterosis in hybrid. Heterosis or hybrid vigor is seen when the hybrids outperform the parental species in certain characteristics such as fertility, developmental speed or biomass (Birchler et al, 2010). In *Drosophila* cases of increased fertility in hybrids have been reported (Gowen and Johnson, 1946, Vetukhiv and Beardmore, 1959, Fry et al, 1998). Similarly, both reciprocal hybrid females in our study clearly demonstrated heterosis in regards to egg deposition and egg hatching. As mentioned previously in both of our fertility estimate experiments we found the average oviposition for both reciprocal females to be significantly higher than either parental species throughout the 10 days (Fig. 3.2, Fig. 3.5, Table 3.1). Reciprocal hybrids also show an asymmetrical level of heterosis in egg hatching. In both fertility experiments either of *F1(sm)* female backcrosses display higher percentage of eggs hatched than the parents. These values were significantly higher when *F1(sm)* females were mated with mated with males multiple times (Fig.3.6). However, *F1(ms)*♀ hybrids only showed heterosis in the percentage of eggs hatched when backcrossed to *mau.01♂* and only when females were mated with males multiple times (Fig. 3.6). Hybrid females also demonstrated heterosis in their respective ovariole count over time. Although, the ovariole numbers were significantly higher in *F1(ms)*♀ than either of the parental species (Fig. 3.7; Table 3.3).
Furthermore, based on the significant differences observed in egg deposition, as well as the differences seen in ovariole numbers between the reciprocal hybrids females, it is clear that maternal effects play a big role in the reproductive fitness of hybrid females. It might even be said that maternal effects are responsible for the heterotic response seen in egg laying in these hybrids. In addition due to the discrepancy seen in egg hatching between the reciprocal hybrids it is equally likely that maternal effects play a role in inducing heterosis in this aspect of fertility in the hybrids as well. Although when looking at heterosis observed in egg hatching of reciprocal hybrid females, one must also consider the potential contributions of the genotypes of the zygote itself to the phenomenon as well.

One last point to consider is what these results show in terms of rate of evolution of speciation in hybrid females. Sexual traits which play major role in speciation have been shown to diverge faster in closely related species. As a result these traits are also expected to show greater morphological differences in inter-specific hybrids due to incompatible gene interactions. This has been shown to be true regarding speciation in hybrid males (Civetta and Singh, 1998). Furthermore sex and reproduction related (SRR) genes have also been shown to evolve faster than genes expressed in other tissues. As well, studies show that SRR genes evolve more slowly in females than in males (Haerty et al, 2007). However our results show that in the case of D. simulans and D. mauritiana female hybrids egg production, a female sexual trait, appears to behave more like a non-sexual trait. That is, if rates of evolution were as fast in females as what is seen in males, then the reciprocal differences in egg deposition in F1 females should not have manifested in the form of heterosis. Thus our result corroborates with the previous findings on the rate of evolution of speciation in females. What it also seems to signify however is that maternal effects play a crucial role in reduction of the rate of evolution of speciation in F1 females. The potential for maternal effects to accelerate or deter evolutionary response to selection was previously described theoretically (Kirkpatric and Lande, 1989). However our findings here together with similar recent finding on maternal effects potentially inducing heterosis in longevity in D. melanogaster hybrids support this
theory (Vaiserman et al, 2013). Despite this evidence also suggests that the effects of heterosis on fertility in the hybrids are brief. Sterility has been observed in F2 backcross females who were homozygous for the X chromosome of *D. simulans* and had inherited the autosomes from *D. mauritiana* (Davis et al, 1994).

4.3 Conclusion and Future Directions

In conclusion our results have shown that the reciprocal hybrid females from crossing *D. simulans* and *D. mauritiana* are not only fully fertile but that they also show heterosis. Furthermore the heterosis observed can be attributed to the presence of maternal effects in between these hybrids. The reciprocal hybrids also show differences in ovariole number compared to each other as well as compared to the parental species, with F1(ms) females having significantly higher ovariole numbers than the parental females. Lastly although the ovariole numbers in hybrids decreased with age, the morphology and structure of their ovaries was still typically larger than the pure species over time mainly due to heterosis. Our findings seem to suggest that maternal effects can act in the form of evolutionary brakes slowing down the process of speciation in females by inducing heterosis and while the brake may only be temporary it is enough to help slow down speciation in females considerably. This also supports the theory of male sex drive, which suggests that speciation is on the large part driven by males (Kulathinal and Singh, 2005). Thus signifying the important role maternal effects plays in slowing down the process of speciation in *D. simulans* and *D. mauritiana* female hybrids.

What is more our results open up many avenues for investigation both regarding a broader study of fertility in female hybrids and their respective role in speciation. Our study only looked at F1 females from *D. simulans* and *D. mauritiana* species pair. But would these findings also apply to other similar species pairs as well? For instance a study can be performed with *D. mauritiana* and *D. sechellia* species since their cross also provides fertile females and sterile males (Lachaise et al, 1986). If the results from these two species pairs differ it would be essential to investigate why that is the case. Furthermore our own study was restricted to egg laying and egg hatching in the F1 reciprocal hybrid females. Identifying the state of viability and fertility in both directions
of the cross in the F2 could be the next step. As mentioned we observed reduction in fertility in $F1(ms)♀ \times sim.169♂$. Classifying why this reduction occurs and whether it is problem of fertilization or due to genetic incompatibility in the F2 would be useful. Lastly further studies on how maternal effects can produce heterosis would be invaluable.
REFERENCES


Plumb, C. S. (1920). *Types and breeds of farm animals*. Ginn, Boston,


**Supplementary Figure 1** General picture of reproductive system in mature female *Drosophila* (Ogienko et al, 2007).
**Supplementary Figure 2** Scheme of *Drosophila* germarium. For details please refer to section 1.5. (Ogienko et al, 2007)
**Supplementary Table 1** Reproductive relationship between four closely related species of *Drosopila melanogaster* complex. Table modified from (Lachaise et al, 1986).

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<th>mauritiana</th>
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<td>----</td>
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**Supplementary Table 2** Summary of trial numbers and results for crosses in fertility estimate-one time mating experiment.

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<tr>
<th>Female (♀) x Male (♂)</th>
<th>Total Trials Tested</th>
<th>Mated</th>
<th>Grand Total of Eggs Laid</th>
<th>Grand Total of Eggs Hatched</th>
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<td>Total</td>
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<td>10</td>
</tr>
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<td>17</td>
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</table>
**Supplementary Table 3** Summary of trial numbers and results for crosses in fertility estimate-multiple matings experiment.

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<th>Female (♀) × Male (♂)</th>
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<th>Grand Total of Eggs Laid</th>
<th>Grand Total of Eggs Hatched</th>
<th>% of Eggs Hatched</th>
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<tr>
<td></td>
<td></td>
<td>Total</td>
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<td>Kept</td>
</tr>
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<td><em>sim.169</em> <em>sim.169</em></td>
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<td>13</td>
<td>2</td>
<td>11</td>
<td>367.2</td>
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Supplementary Figure 3. Range of morphologies of ovaries of pure species and reciprocal hybrid females over time. All images were taken using dark field (DF) setting under 2x magnification using Leica M165 FC fluorescence microscope and mounted camera. All images are to the same scale bar of 1mm. Abbreviations: F1(sm) ♀, F1 female progeny from the cross sim♀ x mau♂; F1(ms) ♀, F1 female progeny from the cross mau♀ x sim♂. A) Ovaries of D. simulans.169, D. mauritiana.01 and F1(sm) and F1(ms) females were dissected on five days post eclosion. All females were mated one day prior to dissection.
Supplementary Figure 3 Continued, B) Morphologies of ovaries of pure species and reciprocal hybrid females at 10 days post eclosion. All females were mated one day prior to dissection.
Supplementary Figure 3 Continued, C) Morphologies of ovaries of pure species and reciprocal hybrid females at 15 days post eclosion. All females were mated one day prior to dissection.
Supplementary Figure 3 Continued, C) Morphologies of ovaries of pure species and reciprocal hybrid females at 20 days post eclosion. All females were mated one day prior to dissection.