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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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POSTZYGOTIC ISOLATION IN *DROSOPHILA SIMULANS* AND *D. MAURITIANA*

(Spine title: Species Isolation in *Drosophila simulans* and *D. mauritiana*)

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by

Christopher Dickman

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
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Postzygotic Isolation in *Drosophila simulans* and *D. mauritiana*

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Abstract

The study of speciation requires examination of barriers that produce and maintain species separation. Using *Drosophila simulans* and *D. mauritiana*, this thesis focuses on post-zygotic isolating mechanisms, which occur after the formation of interspecies hybrids. This study aims to examine the genetic causes of male hybrid sterility and decreased hybrid female lifespan. Quantitative trait locus (QTL) mapping using flies with an attached-X chromosome, identified seven autosomal QTLs that contribute to hybrid sterility. Separately, reduction in hybrid female lifespan was noted for females bearing an attached-X chromosome and was more severe in individuals who were mated. This reduction is caused by a recessive factor on the X chromosome interacting with a dominant autosomal factor. This study is the first to create a hybrid sterility QTL map in *Drosophila simulans* and *D. mauritiana* and also succeeded in characterizing the understudied phenomenon of reduced hybrid lifespan in this species pair.

Keywords

Speciation, Post-zygotic isolation, *Drosophila simulans, Drosophila mauritiana,* QTL mapping, composite interval mapping, multiple interval mapping, lifespan, cost of mating, attached-X chromosome.

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List of Abbreviations

ACPS: Accessory Gland Proteins

BSC: Biological Species Concept

CIM: Composite Interval Mapping

FC: Florida City

LOD: Log of Odds

PCR: Polymerase Chain Reaction

QTL: Quantitative Trait Locus

RFLP: Restriction Fragment Length Polymorphism

SYN: Synthetic

VE: Vitelline Envelope

VERL: VE Receptor for Lysine

Chapter 1

1 Literature Review: Speciation

1.1 Summary

The study of speciation is often focused on the mechanisms by which species become reproductively isolated. Species can be isolated due to barriers that occur before zygote formation (pre-zygotic isolation) or after zygote formation (post-zygotic isolation). In this chapter I review the types of species isolating barriers, and I critically examine different models of reproductive isolation, such as the Dobzhansky-Muller model, which attempts to explain how reproductive barriers evolve. In order to get to some understanding of the molecular basis of speciation, I also examine individual genes responsible for maintaining species separation, as well as how these genes are discovered. Lastly, I discuss genetic mapping methods, such as introgression and quantitative trait locus (QTL) mapping, and then present a functional analysis of genes that are implicated in contributing to reproductive barriers. An interesting observation of surveyed mapping studies is that many of these genes are under positive selection, which would suggest that only a subset of fertility or viability genes contribute to hybrid dysfunction.

1.2 Introduction

One of the fundamental concepts of evolution concerns how species diverge into discrete groups; this is the study of speciation. In the discussion of speciation, it is useful first to define what a species is. The Biological Species Concept (BSC) defines a species as a population comprising of organisms that are unable to mate and produce fertile offspring with other populations when given the opportunity (Mayr 1942). The BSC is possibly the most widely accepted definition of species (Coyne and Orr 2004); however, this definition is controversial due to certain drawbacks. One such drawback is that the BSC can only be applied to sexually reproducing species, and thus cannot describe a large portion of organisms, including all prokaryotes. Another shortcoming of the BSC is that it is only applicable to extant species. As a result, morphological models are required to describe the speciation of populations that are only known from the fossil record. A limitation of these morphological models is that many species, while distinct, are nearly identical in overall body plan. Many organisms are able to interbreed with other populations at a decreased rate, and so do not meet the above definition of a species even though restrictions to gene flow between the two populations keep them mostly separate as evolutionary distinct identities. Organisms that have a decreased level of gene flow between populations, even those that are not completely separated, are still reproductively isolated (Coyne and Orr 2004).

Reproductive isolation mechanisms have been broken down into two main types: pre-zygotic and post-zygotic. Pre-zygotic isolating mechanisms include factors that isolate two populations before the formation of a zygote. This includes behavioral mechanisms that stop individuals from mating, anatomical barriers that make mating

impossible, and mechanisms that occur after mating but interfere with the fertilization of an egg. On the other hand, post-zygotic isolating mechanisms are those that act after successful fertilization, and give rise to dysfunctional interspecies hybrid offspring, or fail to give rise to any offspring at all.

1.3 Pre-zygotic isolation

It is necessary to make a distinction between pre- and post-zygotic isolating factors, as often only one of these factors separates a species pair. Studies conducted with *Drosophila* species have shown that pre-zygotic isolating factors are often present in species that have recently diverged, while post-zygotic isolating factors are present in more distant species (Coyne and Orr 1989). Some species pairs experience only one form of reproductive isolation, either pre- or post-zygotic (Coyne and Orr 1996; Kozak *et al.* 2012). This suggests that pre- and post-zygotic isolating mechanisms have a different genetic basis, *i.e.* they are controlled by different genes and are capable of evolving separately.

A classic example of pre-zygotic isolation involves males of one species being poor courters of the females of another species. Among *Drosophila melanogaster, D. simulans* and *D. sechellia*, each has a specific courtship song which males create through vibrations of their wings. When a mute male attempts to mate with a female while a recording of a conspecific courtship song is played, mating takes place more rapidly and more often than when accompanied by a recording of interspecific song (Ritchie *et al.* 1999). Although in a laboratory setting *Drosophila* females were still willing to mate with individuals accompanied by a recording of interspecific song, it is likely that this

would cause a pronounced decrease in gene flow in the wild when females have the opportunity to mate with more than one male. One component of variation of the song produced by males of different *Drosophila* species is caused by a gene called *period* (Kyriacou and Hall 1980).

One subclass of pre-zygotic isolation involves post-mating pre-zygotic barriers. A classic example is gametic incompatibility. For example, in abalone from the genus *Haliotis* sperm produce a protein called lysin, which is used by the sperm to create a hole in abalone eggs; the holes allow passage of sperm through the vitelline envelope (VE) surrounding the egg (Vacquier *et al.* 1990). The receptor for lysin is called VERL (VE receptor for lysine) and is species specific, such that fertilization occurs at a much higher rate among conspecific gametes than among heterospecific gametes (Swanson and Vacquier 1997).

1.4 Post-zygotic isolation

Post-zygotic isolation occurs when there is dysfunction, such as sterility or inviability of the hybrid offspring. A well-known example of this is the mule, which is the offspring of a male donkey and a female horse. Mules are sterile, and therefore, unable to act as an intermediate to pass genes between horses and donkeys. Another subclass of post-zygotic isolation is hybrid inviability, which is seen when two species are able to produce a zygote that does not grow to maturity. As a result, F_1 individuals are also unable to produce offspring and cannot serve to pass genes from one species to another through backcrossing.

Post-zygotic isolation could be the result of a mutation at a single locus, where an allele of one species interacts with its homolog in the other species when they are combined in a hybrid. One limitation of this theory is that it would require the mutant allele to pass through an individual that is either sterile or inviable (Orr 1997). Consider a population with genotype '*AA*' and another with genotype '*aa*.' Genetically based speciation could result if '*Aa*' hybrids are sterile or inviable. However, the mutant allele '*a*' would have to arise in the heterozygous state '*Aa*', causing sterility or inviability in the individual that first acquired the mutation, and therefore, preventing the allele from being passed on to future generations. This situation, however, could occur if the ancestral population possessed a third allele 'A^*' that mutated independently, in the derived populations, to '*A*' and '*a*', respectively. As this would require multiple, independent mutations at the same locus, which is improbable, multi-locus models have received more attention (Orr 1995).

Bateson (1909), Dobzhansky (1937), and Muller (1942) independently theorized that hybrid dysfunction was caused by the interaction of a mutated allele at one locus with an allele, at another locus, that is incompatible with the first, as illustrated in Figure 1.1. A more complex model would involve interactions at three or more loci. This idea seems to be supported by work in *Drosophila*. Cabot *et al.* (1994) used X chromosome introgressions between *D. mauritiana* and *D. simulans* that introduced genetic material from one species into the genome of the other, and identified three factors (genes) that could cause sterility jointly but not separately.

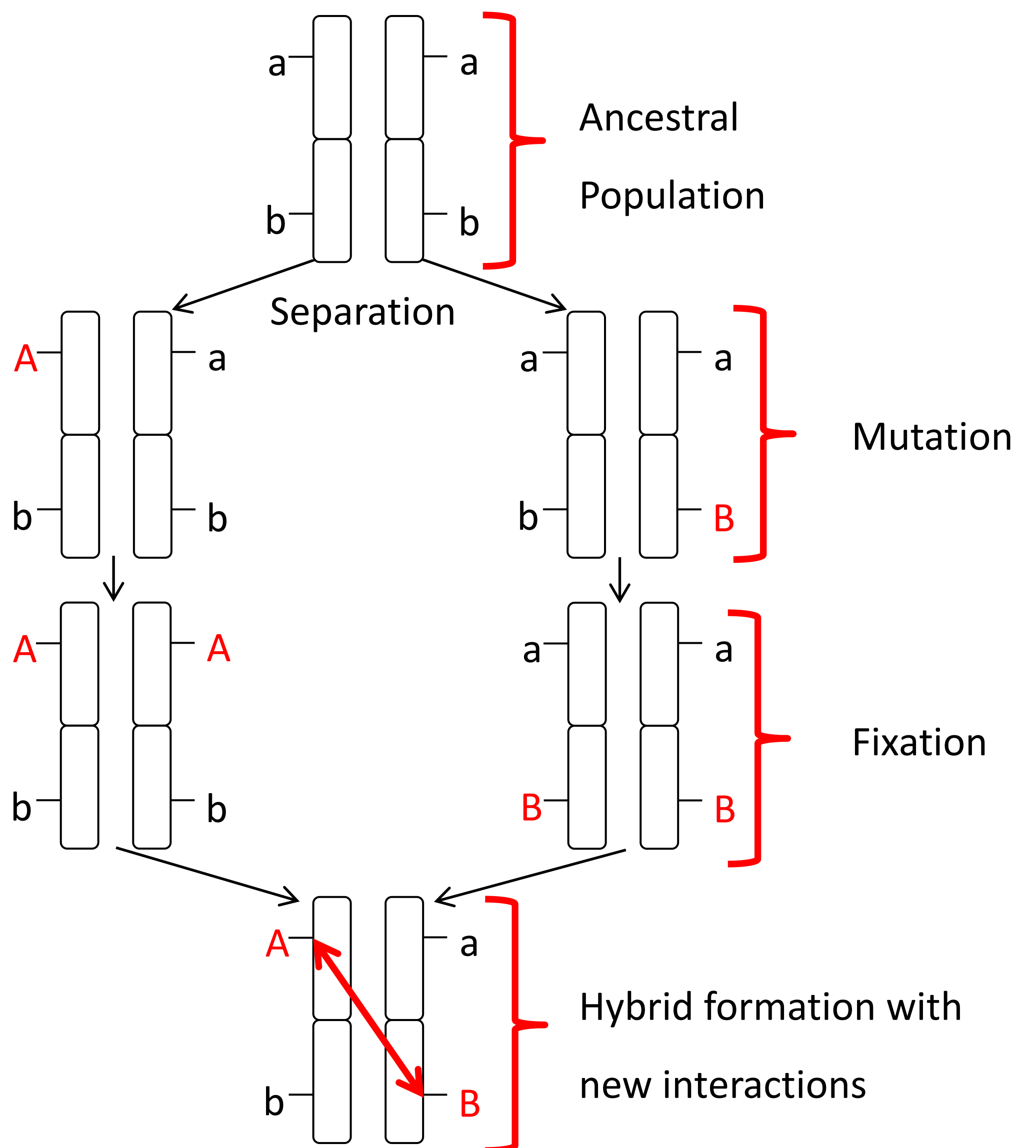

Figure 1.1 Dobzhansky-Muller model. This model proposes the development of hybrid incompatibilities from an ancestral population with genotype *aabb* separating into two populations. Each population has a mutation at a different locus, becoming *Aabb* and *aaBb*. The mutant alleles later become fixed throughout each population. Incompatibilities between the new alleles *A* and *B* could result in reproductive isolation of the two populations. (Adapted from Wu and Ting 2004).

Haldane (1922) noticed that, when two species of organisms interbreed and produce an F_1, often one of the sexes is sterile, inviable, or uncommon. Moreover, the affected sex is more commonly the “heterozygous” or heterogametic sex. In mammals and fruit flies, the male sex is heterogametic, as males possess an X and a Y chromosome, whereas females possess two X chromosomes. In *Drosophila* species, for example, the divergence time between parental species is greater when hybrids are inviable or sterile for both sexes, compared to cases where hybrids of only one sex are sterile or inviable; the affected sex is usually male (Coyne and Orr 1989; Coyne and Orr 1997). There are also many species where the female is the heterogametic sex, such as birds and butterflies, where females have a Z and a W chromosome. In these species, the interspecies hybrid female is more often sterile or inviable (Presgraves 2008; Lijtmaer *et al*. 2002). This also holds true for the hemizygous sex in species such as grasshoppers, where males have one X chromosome and females have two (Haldane 1922). Both heterogametic and hemizygous individuals have only one allele for genes located on the sex chromosome. This is thought to underlie the asymmetric fertility associated with hybrid dysfunction, which is known as “Haldane’s Rule.”

The rate at which different types of incompatibilities arise appears to be different for different types of post-zygotic isolation. Wu (1992) developed a model to show that hybrid sterility appears to evolve more quickly than hybrid inviability. Thus, hybrid sterility arises first in heterogametic individuals, followed by hybrid inviability in heterogametic individuals and ultimately by sterility and inviability in homogametic individuals.

Turelli and Orr (1995) proposed the Dominance Theory as a potential explanation for the genetic basis for Haldane's Rule. The theory states that genes that are located on the X (or W) chromosome can contribute to speciation in homogametic individuals only if they are dominant, whereas every heterogametic individual will be affected regardless of dominance. In other words, genes on the hemizygous sex chromosome will be 'unmasked' in the heterogametic sex (Turelli and Orr 1995). A homogametic individual would have twice as many potential speciation alleles, and therefore, would be expected to contradict this theory by being affected unless speciation genes were on average recessive. Orr (1993a) proposed that most genes contributing to hybrid dysfunction are likely to be recessive as hybrid dysfunction genes tend to be caused by loss of function mutations.

The Snowball Effect theory attempts to ascertain the rate at which all types of Dobzhansky-Muller incompatibilities arise (Orr 1995). The theory suggests that the rate at which incompatibilities arise increases proportionally to the square (or greater) of divergence time; this is because each new mutation has a potential incompatibility with all of the other loci that have experienced divergence, and one must add the potential incompatibilities of previously existing mutations. As each new incompatibility is added to the previously accumulated ones, the number of loci involved is therefore said to 'snowball.' This theory does appear to be true in *D. melanogaster/D. simulans* and *D. melanogaster/D. santomea* hybrids (Matute *et al.* 2010), but not all studies have supported this theory. For example, Lijtmaer *et al.* (2003) examined species pairs with increasing separation time and showed that, over time, the rate of post-zygotic isolation evolves linearly. This would suggest that Dobzhansky-Muller incompatibilities that

evolve early in the process of speciation have a disproportionate effect on fitness compared to incompatibilities that arise later. For example, incompatibilities arising after hybrid sterility is established would not be able make an individual more sterile than it already is. Further examination of the Snowball Effect theory has been hindered by the fact that there are few genetic model organisms capable of making hybrids with multiple species. It is therefore difficult to show a comparison between the number of incompatibilities a species has with multiple sister species of different divergence times.

Mutations in a relatively small number of genes are not the only possible cause of post-zygotic isolation. Species that have been separated long enough to have undergone major rearrangements of their chromosomes, including changes in chromosome number or translocations, could give rise to hybrids that lack a large number of genes. The yeast species *Saccharomyces cerevisiae* and *S. mikatae* are normally unable to produce a fertile F_1, in part due to a series of translocations among their ancestors. In a study by Delneri *et al.* (2003), the researchers induced a reconfiguration of the *S. cerevisiae* genome to make it collinear *i.e.* identical in karyotype with that of *S. mikatae,* and partially rescued fertility of the hybrid offspring, which produced a large portion of viable spores. The authors concluded that the translocations did not drive the speciation of *S. cerevisiae* and *S. mikatae* because of a lack of correlation between translocation events and the sequence based phylogeny*;* however, the results are still notable as they show that translocations can maintain reproductive isolation. It is interesting to note that across many species pairs that experience hybrid incompatibilities, only a fraction have major rearrangements of the genome, while the majority of species pairs have collinear chromosomes (White 1969).

Consequently, the chromosomal differences cannot be regarded as the prevalent cause of speciation.

Another proposed mechanism of hybrid sterility is an incompatibility between centromeres and their binding proteins. Centromeres are known to evolve quickly, as are the proteins that bind them (Malik and Henikoff 2001). Centromere binding proteins are important during meiosis to provide an attachment for meiotic spindles. Henikoff *et al.* (2001) proposed a mechanism by which evolution of the centromere in two populations leads to the co-evolution of centromere binding proteins (such as Centromere identifier; Cid). Hybrids between these two populations could lack the proteins necessary to segregate the chromosomes during meiosis, leading to a failure in gamete production. This model could also explain Haldane's rule, because heterogametic chromosome pairs already have the most dissimilar centromeres, which would cause the dysfunction to be magnified (Henikoff *et al.* 2001).

Hybridization does not always lead to a decrease in fitness. In fact, it has occasionally been shown to increase fitness. An often cited example is that of *Artemisia tridentate,* a sagebrush plant with two sub-species, *A. t. tridentata* and *A. t. vaseyanai,* which occupy lowland and mountain habitats, respectively. The hybrids of these two species are able to exploit the intermediate altitude regions better than the parentals (McArthur *et al.* 1988). Hybridization can sometimes even give rise to new species. Hybrid speciation has occurred in sunflowers of the genus *Helianthus,* with three hybrid species *H. paradoxus, H. anomalus,* and *H. deserticola* being independently formed hybrids of *H. annuus* and *H. petiolaris,* all of which are better adapted to extreme environments than the progenitor species (Rieseberg *et al.* 1991).

1.5 Mapping techniques

To understand the genetic basis of speciation, one must first locate the genes responsible for reproductive isolation. This is complicated by the fact that most of the methods discussed below require the examination of individuals that are only partially reproductively isolated and therefore still capable of exchanging genes. There are several different types of gene mapping, each with its own benefits and drawbacks. Introgression mapping involves the insertion of small fragments of DNA from one species into another. This method has identified *Odysseus-site homeobox* (*OdsH*), a gene contributing to F_2 hybrid sterility in *D. simulans* and *D mauritiana* crosses (Perez *et al.* 1993). Recombination mapping is similar. It involves examining crossing-over between a series of markers to determine where the genetic material affecting by the examined phenotype is located. Deficiency mapping involves the use of certain stocks of a species that have a hemizygous deletion in a known span of a chromosome. Only one allele in the deficiency region is present and thus able to affect the corresponding phenotype. This technique is used to unmask genes that may act recessively when an F_1 is created. A given phenotype is tested with several *Drosophila* lines that have deficiencies in the same area to narrow down the region of interest and to reduce the possible effect of differing genomic backgrounds. A downside is that deficiencies are only available in *D. melanogaster,* and to a lesser extent, in *Caenorhabditis elegans.* Despite this drawback, deficiency mapping has been successfully used to discover genes that maintain species separation, such as *Nucleoporin 98-96* (*Nup 96*), a nuclear pore protein that adaptively diverged in *D. melanogaster* and *D. simulans* and contributes to hybrid inviability (Presgraves *et al*. 2003).

It is possible to identify genes that contribute to species isolation using data that have already been collected for other purposes. The human genome has been intensively studied for genes that cause disease, and thousands of mutations have been identified that are known to be lethal in humans (Jimenez-Sanchez *et al.* 2001). Kondrashov *et al.* (2002) took advantage of this wealth of information to compare, across species, SNPs that were lethal in humans but normal or adaptive in other species. The study examined 32 human genes with homologues in a variety of other species and found that all but 8 had diverged mutations that were pathological in humans but not in other species (Kondrashov *et al.* 2002). These data suggest that these genes are capable of creating functional proteins but caused disease through their interaction with other loci, in essence a Dobzhansky-Muller incompatibility. The likelihood that a gene caused an internal incompatibility was independent of the divergence time between humans and other species, including other primates (Kondrashov *et al.* 2002). This is in contrast to studies in organisms with closely related sister species so it would seem that there is a plateau in evolutionary distance at which incompatibilities are no longer more likely to evolve.

1.5.1 Quantitative trait locus mapping

Quantitative trait locus (QTL) mapping is similar to recombination mapping with the exception that it can be used to examine multiple regions of the genome at the same time. To determine the location of recombination events, QTL mapping needs markers such as SNPs, microsatellites, or, occasionally, visible markers. These data are then analyzed using one of a variety of statistical models (Zeng 1993; Kao *et al.* 1999; Yi and Xu 2000) and computer software such as QTL cartographer (Basten *et al.* 1999). QTL mapping is

well suited for analyzing entire genomes for multiple loci that may act epistatically, allowing for the detection of Dobzhansky-Muller incompatibilities, which occur when two or more genes interact to cause hybrid dysfunction. The effectiveness of QTL mapping is influenced by the number and spacing of molecular markers, as well as the sample size and heritability of the trait (Zeng 1993). Loci with large effects are easier to detect and for this reason it has been hypothesized that there is a bias towards identification of large effect loci as contributing to speciation (Rockman 2012).

QTL mapping is greatly assisted by the presence of genome sequence data, with *D. melanogaster* being sequenced in the last twelve years (Adams *et al.* 2000). This allows for the more rapid creation of molecular markers such as RFLPs, and also for a more thorough analysis of identified QTL for candidate genes. In part due to sequence availability, the number of studies featuring QTL mapping has increased in the last several years (Rockman 2012).

A weakness of QTL and other methods of mapping is that once a region or gene is identified as contributing to hybrid dysfunction, it is difficult to determine which genes were involved in the process of speciation, as there are no ancestral individuals that can be examined when the species pair was less diverged. Although two species may have a hundred genes that are capable of causing hybrid sterility, the most important in the context of speciation is the first to diverge between species pairs, the first that is capable of causing complete sterility.

1.6 Genes that cause hybrid dysfunction

Several genes for hybrid sterility have been identified (see Table 1.1). It is useful to examine how these genes arose and see if there are any trends in how they cause dysfunction both in terms of the genes' pathway and the molecular function of the individual gene products. Most of the genes listed in Table 1 have been found in rapidly reproducing model organisms and so give a limited picture of the genetic basis of hybrid dysfunction as it applies to all species. Also of note is which generation of hybrid these genes affect; many only cause dysfunction in individuals where the gene has been homozygously introgressed in the background of another species. This is not the genetic combination present in the F_1 generation and so many of these genes only explain sterility in later generations. Section 1.6 will provide an overview of some of the most notable hybrid dysfunction genes as well as examine any similarities in their evolutionary history and genome ontology, *i.e.* their molecular function, the cellular component they act in as well as the biological processes they affect.

Gene transposition has been shown to be capable of causing hybrid sterility even in individuals that do not have major chromosomal re-arrangements (Masly *et al.* 2006). Some hybridizations of *D. melanogaster* and *D. simulans* produce sterile males due to a translocation. The gene *JYalpha* was transposed from the 4th chromosome, where it is located in *D. melanogaster*, to the 3rd chromosome of *D. simulans* during the divergence of the two species (Masly *et al.* 2006). Hybrids that were homozygous for the 4th chromosome of *D. simulans* were sterile as they lacked a Na^+/K^+ ATPase necessary for sperm production (Masly *et al.* 2006). Individuals that were heterozygous for the 4th

chromosome, as well as flies that were transgenically altered to include *D. melanogaster JYAlpha* were fertile, showing that this gene is capable of rescuing sterility in hybrids that otherwise lack a copy of this gene (Masly *et al.* 2006). It is worth pointing out that this gene would only affect the sterility of later-generation individuals that entirely lacked a copy of *JYAlpha*, and so does not affect F_1 hybrids. This gene would not be expected to have as large of a contribution to the restriction of gene flow as a gene capable of causing sterility in an F_1.

Table 1.1 List of post-zygotic isolating genes. This table shows genes known to contribute to hybrid sterility or inviability, as well as the species pair affected by each gene. 'Capable of acting Dominantly' refers to the ability of the sterility allele to have an effect in a heterozygous state, 'NA' is used when a gene effecting male sterility is located on the X chromosome and therefore a dominant interaction would not be possible (adapted from Presgraves 2010).

Gene Name	**Symbol**	**Phenotype**	**Species Pair**	**Putative Normal Function**	**Sex linked**	**Capable of Acting Dominantly**	**References**
ATPase expression 2	*AEP2*	Sterility	*Saccharomyces bayanus/ Saccharomyces cerevisiae*	Translational regulation of OLI1	No	No	Lee *et al.* 2008
Oligomycin resistance 1	*OLI1*	Sterility	*S. bayanus/ S. cerevisiae*	ATP-synthase subunit	No	No	Lee *et al.* 2008
JYalpha	*JYalpha*	Sterility	*Drosophila simulans/ D.melanogaster*	$Na^{+}K^{+}$ATPase	No	No	Masly *et al.* 2006
Overdrive	*Ovd*	Sterility	*Drosophila pseudoobscura bogatana/ D. pseudoobscura pseudoobscura*	DNA binding	Yes	NA	Phadnis and Orr 2009
Pr domain containing 9	*PRDM9*	Sterility	*Mus musculus musculus/ M. musculus domesticus*	Meiotic histone H3 methyltransferase	No	Yes	Mihola *et al.* 2009

Odysseus-site homeobox	*OdsH*	Sterility	*D.mauritiana/ D. simulans*	DNA binding	Yes	NA	Ting *et al.* 1998
S5	*S5*	Sterility	*Oryza sativa indica/ O. sativa japonica*	Aspartate protease	No	Yes	Chen *et al.* 2008
SaF	*SaF*	Sterility	*O. sativa indica/ O. sativa japonica*	F-box protein	No	Yes	Long *et al.* 2008
SaM	*SaM*	Sterility	*O. sativa indica/ O. sativa japonica*	Sumo E3 ligase	No	Yes	Long *et al.* 2008
Histidinol-phosphate amino-transferase 1	*HPA1*	Inviability	*Arabidopsis thaliana* intra-species	Histidine synthesis	No	No	Bikard *et al.* 2009
Histidinol-phosphate amino-transferase	*HPA2*	Inviability	*A. thaliana* intra-species	Histidine synthesis	No	No	Bikard *et al.* 2009
MRS1	*MRS1*	Inviability	*S. cerevisiae/ S. bayanus* OR *S. paradoxus*	Gene splicing of COX1	No	No	Chou *et al.* 2010
Cytochrome c oxidase subunit 1	*COX1*	Inviability	*S. cerevisiae/ S. bayanus* OR *S. paradoxus*	Cytochrome c oxidase subunit	No	No	Chou *et al.* 2010
Altered inheritance rate of mitochondria 22	*AIM22*	Inviability	*S. cerevisiae/ S. bayanus* OR *S. paradoxus*	Lipoate protein Ligase	No	No	Chou *et al.* 2010
Dangerous mix 1	*DM1*	Inviability	*A. thaliana* intra-species	Nucleotide binding immunity protein	No	Yes	Bomblies *et al.* 2007

Zygotic hybrid rescue	*Zhr*	Inviability	*D. melanogaster/ D. simulans*	Unknown (repetitive DNA)	No	No	Sawamura and Yamamoto 1997
Hybrid male rescue	*Hmr*	Inviability	*D. melanogaster/ D. simulans*	DNA binding	Yes	No	Barbash *et al.* 2003
Lethal hybrid rescue	*Lhr*	Inviability	*D. simulans/ D. melanogaster*	DNA binding	No	No	Brideau *et al.* 2006
Nucleoporin 96	*Nup96*	Inviability	*D. simulans/ D. melanogaster*	Nuclear pore	No	No	Presgraves *et al.* 2003
Nucleoporin 160	*Nup160*	Inviability	*D. simulans/ D. melanogaster*	Nuclear pore	No	No	Tang and Presgraves 2009

Studies that seek to identify genes for hybrid sterility have primarily been able to locate genes that have an effect when homozygous. A well-known example is the gene *OdsH,* a homeodomain protein-coding gene found on the X chromosome of *D. mauritiana* (Ting *et al.* 1998). Homeodomains are found most commonly in transcription factors and so it is reasonable to conclude that *OdsH* plays a role in genetic regulation. Knockout flies that are deficient for *OdsH* have slightly reduced fertility, but this effect is only noticeable at a young age (Sun *et al.* 2004). When this allele, is co-introgressed with a linked region into the background of *D. simulans,* sterile males are produced (Perez 1995). It is interesting that a gene would have a small effect on fertility in a pure species individual, but a large effect in a hybrid; this would likely be due to epistatic effects of the gene in a foreign background. It has not been shown that this gene is capable of causing sterility in an individual with heterozygous autosomes, and therefore, it cannot be concluded that the gene is responsible for some of the sterility seen in F_1 individuals.

Looking at the above data raises the question: is the Dominance Theory supported by the wealth of genetic analyses completed? As reviewed by Coyne and Orr (2004), a prediction of this model is that a homogametic F_1 would become sterile or inviable when the X (or Z) chromosome was homozygous, such that all alleles on the X would be expressed regardless of dominance. Studies in *Drosophila* using unbalanced females do seem to support the model. Coyne (1985) tested female sterility in *D. simulans*/*D. mauritiana* and *D. simulans*/*D. sechellia* unbalanced F_1s that had inherited both X chromosomes from one parent, and found that these individuals were fertile, like normal F_1 females. This was subsequently shown in other species pairs (Orr 1987), and makes sense given that a gene that would affect male sterility would not necessarily affect

female sterility. A gene that affected viability, however, would be expected to affect individuals of both sexes. In another study that used unbalanced females (containing both X chromosomes from one species) in the species pairs *D. simulans/D. melanogaster* and *D. simulans/D. teissieri*, it was found that the unbalanced F_1 females do become inviable when in possession of homozygous X chromosomes (Orr 1993b). The unbalanced female tests only support the dominance theory with regards to inviability, but not sterility.

One phenomenon associated with post-zygotic isolation that has received a lot of attention is the large X effect, which refers to the propensity of genes located on the X chromosome to cause hybrid dysfunction. For example, in *D. mauritiana,* a gene located on the X chromosome is approximately three times more likely to cause hybrid sterility than a gene located on an autosome (Masly and Presgraves 2007). The evolutionary basis for the ‘large X effect’ is unknown, but a possible cause involves difficulties in X inactivation during sperm development. Also, there is divergence in the mechanism of dosage compensation between the sexes, as males require some X chromosome genes to be hyper-transcribed (Masly and Presgraves 2007). This could make genes on the X chromosome sensitive to disruptions in gene regulation, especially in males, which could contribute to Haldane’s Rule.

It is of interest that genes that have been shown to cause hybrid dysfunction appear to be experiencing positive selection - they have a higher ratio of non-synonymous to synonymous mutations than would be expected by chance. *Nup96* codes for a nuclear pore protein that contributes to inviability. It is generally conserved across eukaryotes and has been shown to be under positive selection in both *D. simulans* and *D. melanogaster* (Presgraves *et al.* 2003a). The same was found with *OdsH* (Ting *et al.*

1998) and *Hmr* (Barbash *et al.* 2003). From table 1.1, 7 out of 13 genes tested were found to be experiencing positive selection (Barbash *et al.* 2003; Bikard *et al.* 2009; Bomblies *et al.* 2007; Brideau *et al.* 2006; Chou *et al.* 2010; Lee *et al.* 2008; Masly *et al.* 2006; Mihola *et al.* 2009; Presgraves *et al.* 2003; Phadnis and Orr 2009; Tang and Presgraves 2009; Ting *et al.* 2008). Presgraves (2010) speculates that the high rate of mutation in hybrid dysfunction genes could be due to two reasons. The first is that these genes will only cause an incompatibility after being mutated several times. The second is that only a small fraction of the mutations will cause an incompatibility, and genes with more mutations have a higher chance of not functioning in a hybrid. Future studies may identify which mutations in these genes are causative of the hybrid incompatibility.

Determining whether there are similarities in the function of the genes involved in speciation tells us whether certain pathways or classes of proteins are more susceptible to speciation-causing mutations. *OdsH* (Ting *et al.* 1998), *Hmr* (Barbash *et al.* 2003), and *Ovd* (Phadnis and Orr 2009) all have DNA-binding motifs, consistent with the view that they are transcription factors, and therefore, problems with gene regulation could be a common cause of post-zygotic isolation; however, as mentioned earlier, *Nup96* is a nuclear pore protein and so the phenomenon is not universal. As one would expect, genes involved in male sterility tend to be expressed in the testes rather than acting somewhere else in the body (Bayes and Malik 2009; Mihola *et al.* 2009; Phadnis and Orr 2009). However, genes for inviability have not shown a trend for localization in specific regions. For example, both Nup96 and Nup160 are present in the nucleus of every cell (Presgraves *et al.* 2003a; Tang and Presgraves 2009).

1.7 Conclusions

There has been a great deal of work on the genetic basis of speciation. From the data presented above, it appears that a single proposed cause cannot be identified for the genetic origin of post-zygotic isolation, although clear trends exist. While work in model systems such as certain *Drosophila* species is likely to continue, branching out into an examination of hybrid dysfunction in different clades of non-model organisms will provide insights into the universality of observed phenomena and whether or not models like the snowball effect are the rule or exceptions.

In Chapter 2, I will describe a QTL mapping project that identified loci contributing to hybrid sterility in the species pair *D. simulans* and *D. mauritiana,* that were predicted to exist under the dominance theory. Through the use of special stocks and crossing schemes, I was able to examine the fertility of backcross hybrid males that have inherited their X chromosome entirely from one parental species. Holding the X chromosome constant across all tested individuals allows for the mapping of autosomal genes that are capable of acting in the heterozygous state, as the disproportionate effect of the X chromosome on sterility will be stable. This is unique, as most previous mapping studies have looked at homozygous genes that may not be capable of causing sterility in an F_1.

Chapter 3 discusses my discovery of the phenomenon of reduced lifespan, which affects hybrid females of *D. simulans* and *D. mauritiana*. I also noticed that these females have an increased cost of mating, *i.e.* a greater reduction in lifespan when they are paired with males. This chapter quantifies the lifespan of each population with respect to different genetic combinations, whether these individuals are F_1s or backcrosses, and to

which parental species these individuals are mated. The chapter also discusses the evolutionary implications of hybrid lifespan reduction and cost of mating, a relatively understudied form of post-zygotic isolation.

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Chapter 2

2 Hybrid sterility QTL on the autosomes of *Drosophila simulans* and *D. mauritiana*

2.1 Abstract

Drosophila simulans and *D. mauritiana* are a closely related pair of species that have been previously examined, for the study of hybrid sterility and its genetic basis. Previous studies have focused on methods such as introgression mapping, which cannot distinguish between genes that act dominantly or recessively, and also disproportionally locate genes on the X chromosome, although they have succeeded in identifying some loci capable of causing sterility. Using a crossing scheme involving an attached-X stock of *D. simulans* where females have two X chromosomes fused together, backcross males were created that possessed recombinant autosomes and non-recombinant sex chromosomes. This allowed the heritable variation in phenotype to be solely caused by differences in the autosomes. The dominance theory proposes that sterility is caused by dominant alleles (or incompletely dominant) on the autosomes interacting with recessive alleles of the X chromosome. This study mapped to the autosomes of this species pair seven quantitative trait loci (QTL) that are capable of acting in a heterozygous state, and therefore, capable of acting dominantly. This goes some way towards explaining hybrid sterility seen in the offspring of *D. simulans*/*D. mauritiana* crosses, where only dominant acting genes would be expressed, lending support to the dominance theory.

2.2 Introduction

The study of speciation often focuses on examining modes of reproductive isolation. Reproductive isolation occurs when there is a barrier that prevents two species from producing fit hybrid offspring. Reproductive isolation is either pre-zygotic, and caused by factors that occur before the formation of a zygote, or post-zygotic isolation, where there is a dysfunction within the hybrid offspring of two species. Post-zygotic isolation, which includes hybrid sterility and inviability, is the focus of this chapter.

Both hybrid sterility and inviability reduce an individual's fitness to zero if their effect is complete; however, in determining which has a greater effect on speciation, it is prudent to focus on which factor evolved first. An examination of the work performed on *Drosophila* species indicates that hybrid sterility is ten times more frequent than inviability in interspecies crosses (Bock 1984). This suggests that sterility evolves at a quicker rate than inviability and occurs more often despite the fact that inviability arises more readily as a result of mutation (Cooley *et al.* 1988). From these observations, the idea was proposed that genes involved in hybrid sterility, especially in males, were under selection positive (Wu and Davis 1993).

It is important to look at the possible genetic causes of hybrid dysfunction. One controversial model is the Dobzhansky-Muller model (Bateson 1909; Dobzhansky 1934; Muller 1939). This model can be illustrated using a hypothetical ancestral species with the genotype $A_1A_1B_1B_1$. This genotype could split into two populations that each diverge at a separate locus to acquire the genotypes $A_2A_2B_1B_1$ and $A_1A_1B_2B_2$, respectively. If these genes act epistatically, a hybrid with the genotype $A_1A_2B_1B_2$ may have a genetic incompatibility involving the mutant alleles A_2 and B_2. If hybrids between these two

populations were rare, the derived forms of *A* and *B* would never have come into contact, and therefore, there would not have been a selective pressure to ensure these new alleles functioned together. In the above example, if the alleles causing the incompatibility act dominantly they can underlie post-zygotic isolation in F_1 hybrids, as the alleles in the $A_1A_2B_1B_2$ individual are heterozygous, and therefore, only dominant alleles would have an effect.

On its own, the Dobzhansky-Muller model described above cannot explain Haldane's rule, which states that in interspecies hybrids, when one sex is sterile or inviable, it is more often the heterogametic (XY or ZW) sex. One theory which could explain both hybrid dysfunction and Haldane's rule is the Dominance Theory. Building from the example shown above, if gene '*A*' is located on the X chromosome and gene '*B*' is on an autosome, a hybrid male will be affected regardless of whether or not the '*A*' allele is dominant or recessive, because there is no alternate allele. This would not be true for an XX female, as a dominant allele would mask the expression of a recessive gene. One would expect that females who were homozygous for genes on the X chromosome could also be affected, which was tested in *Drosophila simulans* and *D. mauritiana* hybrids (Coyne 1985) and shown not to be the case. The suggested reason for females remaining unaffected was that genes causing female sterility are different from the genes causing male sterility.

Drosophila simulans and *D. mauritiana* constitute a species pair that is often used to study hybrid sterility. The benefit of these species is that they are closely related to the well-studied *D. melanogaster*, to which they are almost identical in both outward appearance and genetic composition. *D. melanogaster*, however, is unable to interbreed

with any of its sister species to produce fertile offspring. In evolutionary terms, *D. simulans* and *D. mauritiana* are relatively recently diverged, having separated about 250 thousand years ago; whereas, *D. simulans* and *D. melanogaster* diverged approximately 3 million years ago (Kliman *et al.* 2000). The genome of *D. simulans* has been sequenced, providing a great deal of molecular tools, including information on molecular markers for genotyping, such as microsatellites and indels. These features taken as a whole make *D. simulans*/*D. mauritiana* one of the most commonly used species pairs to perform genetic analysis on post-zygotic isolation.

Drosophila simulans and *D. mauritiana*, when crossed, produce sterile F_1 males and fertile F_1 females. Previous studies in this species pair have identified a gene, *OdsH*, that is capable of causing sterility in male hybrids between the two species (Perez *et al.* 1993; Ting *et al.* 1998). This X-chromosome gene will only cause sterility in homozygous introgression lines, and therefore, does not add support to the Dominance Theory. Although undoubtedly contributing to sterility in the recessive condition, *OdsH* is not sufficient to explain the sterility seen in F_1 individuals in this species pair. Previous studies that have attempted to map genes capable of causing sterility in the F_1 generation of interspecies *Drosophila* crosses have shown that the majority of QTL localize to the X chromosome, and that these genes have a disproportionately large effect on the sterility phenotype (*e.g.*, Moehring *et al.* 2006b). The gene *Overdrive* (*Ovd*) in *D. pseudoobscura*, for example, also localizes to the X chromosome and has no identified interactor, although *Ovd* is capable of causing sterility in an F_1 between *D. pseudoobscura pseudoobscura* males and *D. pseudoobscura bogotana* females. However, it is possible that the large sterility effect of genes on the X chromosome hinders the search for

autosomal interactors by masking their effect in studies that use recombinant individuals, and therefore, the search could potentially progress further if the effect of the X chromosome was held constant. This study bypasses the problem of the 'large X effect' by holding the X chromosome constant, and thus, may improve resolution in the search for sterility loci on the autosomes.

This study uses an attached-X stock and a crossing scheme that will give rise to backcross males that have a non-recombinant X chromosome and a set of recombinant autosomes (Fig. 2.1). Testing backcross individuals that only vary at their autosomes will allow for improved mapping of the number, location, and effect size of interactors on the autosomes. To examine whether the genetic cause of interspecies hybrid sterility varied with respect to backcross direction, F_1 flies were backcrossed to both parental species.

2.3 Materials and methods

2.3.1 Stocks and crosses

D. mauritiana synthetic (SYN; Coyne 1989), *D. simulans* Florida City (FC; Coyne 1989), and a *D. simulans* attached-X line (C(1)RM w/1z^5; provided by D. Presgraves) were used. The attached-X, which is only present in females of the stock, has a mutation in the *white* gene which makes these females have white-colored eyes; males, which have a single non-mutant X chromosome, have red eyes. This assists in confirming stock integrity: if the attached-X becomes disassociated, then white-eyed males and red-eyed females would be observed in the next generation. Flies were kept in an incubator on a 14:10 hour light:dark cycle at 24°C on standard Bloomington medium (Bloomington *Drosophila* Stock Center). Virgin attached-X *D. simulans* females were collected daily,

aged five days, and then crossed with 1-6 day-old *D. mauritiana* males to create F_1s. F_1 females were collected daily and immediately crossed to the parental species males, *D. simulans* FC or *D. mauritiana* SYN. This crossing scheme ensured that backcrosses possessed non-recombinant sex chromosomes while possessing a set of recombinant autosomes (Fig 2.1).

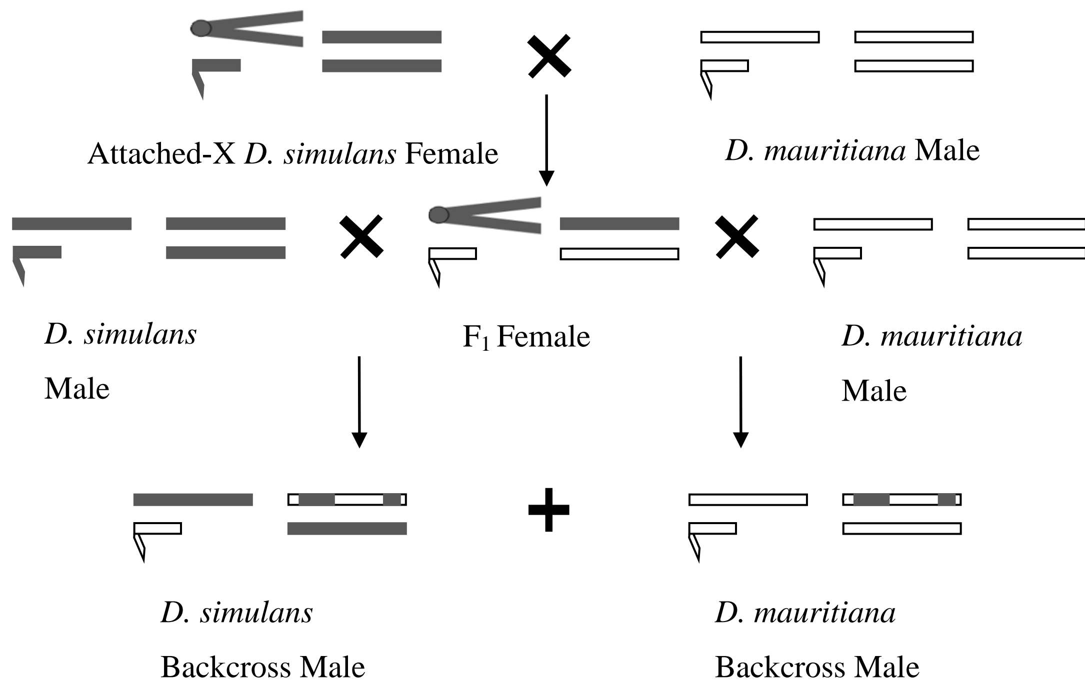

Figure 2.1 Crossing scheme used to obtain backcross males. This diagram represents all homologous pairs of autosomes as a pair of bars on the right for each individual. Sex chromosomes are on the left for each individual, with small hooked bars representing Y chromosomes, longer bars representing X chromosomes, and two joined bars representing the attached-X chromosome. Grey denotes *D. simulans* genetic material and white *D. mauritiana* material. Note that attached-X females also carry a Y chromosome, but remain female due to the mechanism of sex determination in *Drosophila*.

2.3.2 Sperm motility assays

Sperm motility was assayed as a proxy for male fertility. Although it is possible for a male with motile sperm to be sterile, this method has been shown to account for most cases of infertility (Coyne and Charlesworth 1986). Pairing two individuals and measuring if any offspring arise is less reliable, as this could be confounded by female preference. Backcross males from both directions were collected within 10 hours of eclosion and aged for 3 to 5 days to ensure reproductive maturity. The flies were then placed in Biggers–Whitten–Whittingham buffer (Zhang *et al.* 2007) and the testes were removed. The body of the fly, except for the testes, was frozen for later DNA analysis. The testes were gently crushed underneath a glass coverslip and observed under a light microscope using phase contrast. Each individual was scored for the presence of sperm and whether or not sperm was motile. 266 *D. simulans* backcross and 760 *D. mauritiana* males were dissected. Preliminary results showed that it was not possible to count large numbers of motile sperm using this method, and so a scaled scoring system of sperm abundance was not used. Testes were analyzed on whether or not there was any sperm and whether or not any sperm was motile. As a control, 10 three day old males each of *D. simulans* FC and *D. mauritiana* SYN were also assayed for sperm motility.

2.3.3 Genotyping backcross to *D. mauritiana* individuals

Genotyping was completed using microsatellite analysis for 20 markers throughout the second and third chromosomes (Table 2.1). The primers were initially tested on 5 *D. simulans* and 5 *D. mauritiana* flies to ensure that the markers were divergent between the two species, but not polymorphic within each species. The markers on the second chromosome were amplified individually using PCR and run on a 3% agarose gel.

Markers on the third chromosome were amplified using fluorescently-labeled primers in a multiplex PCR reaction and the samples were analyzed using capillary electrophoresis at the Michael Smith Laboratories Nucleic Acid Protein Service Unit (BC, Canada). An X chromosome marker was used to control for contamination of the stocks and test for separation of the attached-X chromosome, which would cause the backcross males to receive an X chromosome from the alternate species. The marker was not used for QTL analysis, as it was only ever inherited from one species per cross.

Table 2.1 List of microsatellite markers. A list of the molecular markers and their primers used for genotyping. Genomic location is relative to the *D. simulans* published genome sequence for the X chromosome and the left and right arms of the second and third chromosomes (2L, 2R, 3L, and 3R, respectively).

Name	Forward primer	Reverse primer	Genomic Location	Source
2L 770	GTGCAGCGCCTTTATGTTTT	TGCTCTCGTTGAAAATGTCG	2L 770995-771172	Dickman C.T.D.
AC0005889	GCGTGGCTGGCATATAG	TAAGCCCCCTCGTGTAATTG	2L 9002412-9002601	(Moehring *et al.* 2004)
2L 11774B	TCCGAGATCCGTGTCTTTCT	CATGTTGCATTTGCCTTGAC	2L 11775227-11775555	McNiven, V.T.K., unpublished data
Su(h)	AACGGCTCACCCCTCGATCC	TACTTCTCCATGGCGTCCCG	2L 14787128-14787318	(Civetta *et al.* 2002)
2L 21651	TCGCACTTTACGAGGTGTTG	AATGCCAGTTCGGATAGTCG	2L 21651886-21652097	Dickman C.T.D.
2R 700	CTGGAACTGTGGGTGGAAAG	CCCATCTCATCTCCCTTCCT	2R 700869-701094	Dickman C.T.D.
Drogpad	GAAATAGGAATCATTTTGAATGGC	AATTAAAAACAAAAAACCTGAGCG	2R 4976473-4976630	(Schug *et al.* 1997)
2R 14938	CACCCTTACCCTGTTCCTCA	GACTTTCCCCTTTTCCTTGC	2R 14938944-14939284	McNiven, V.T.K., unpublished data
2R 15381B	CGGAACCAGCAGAAACTCTAA	TCACAGACCCTCCATTCAAAG	2R 15381226-15381456	Dickman C.T.D.
2R 19158	GCTCACGTTCGTTTATGCTG	CGGTGCAAATTACGACACAG	2R 19158946-19159242	Moehring, A.J., unpublished data
3L 1457	TGGAGAGCGGCGTTCCCCTGTGT	TGGGCCACCTGTGGGCGTGGT	3L 1457712-1457889	Moehring, A.J., unpublished data
3L 3484	GAGGACAGGCGGTACATGAG	TAGTCCGTGGGCAGTAGCTC	3L 3484769-3485091	McNiven, V.T.K., unpublished data

3L 10365	GACCCGAGAGCATTCTTGAG	GTTTCCCTGCCCAAGAGACAATT A	3L 10365945-10366281	McNiven, V.T.K., unpublished data
3L 16008	CCAAGGGGCAGAAATAGGTA	GGAGCAACAATTGCATCAGA	3L 16008277-16008635	Moehring, A.J., unpublished data
3R 697	GGAGATGCCAAACGAAATA	CTCTTTCCGCTCCCCTTA	3R 697841-698111	Moehring, A.J., unpublished data
3R 3880	CCTCCTTGGAATGATCCTCA	ATTATCCAAGTGCGGACGAC	3R 3880676-3881146	Moehring, A.J., unpublished data
3R 4012	CGGGTTAATTGGACTTGCAT	CTGGCCAAGTCGAGAAAAAG	3R 4012692-4013132	Moehring, A.J., unpublished data
3r 17066	GCGATTGTGTGCGAGTGTAT	GGGGGATTTTGTTTGTCATC	3R 17066022-17066224	McNiven, V.T.K., unpublished data
3R 20144	GAACAAGCCGGCATACAGAT	GTTTAGGCACATTTGGATTGGA TT	3R 20145125-20145428	McNiven, V.T.K., unpublished data
3R 23001	TAGCTGCCATCGAGTGTGTC	GTTTTGCGGCTAATGAGAGG	3R 23002040-23002276	McNiven, V.T.K., unpublished data
X 16836	GGGCGGAAAGTAGAGAAGGT	GCCCACTGATTTGGCTATGT	X 16836880-16837168	McNiven, V.T.K., unpublished data

2.3.4 QTL analysis

Quantitative Trait Loci (QTL) mapping was performed in three different ways: 1) using sperm motility as a binary trait, *i.e.*, presence or absence of motile sperm, 2) additionally using an intermediate trait: sperm present but immotile, and 3) analyzing data based on the presence of sperm whether motile or not. QTL were mapped using composite interval mapping (CIM; Zeng 1994). This was done using the computer program QTL cartographer (Basten *et al.* 2004).

Composite interval mapping, like interval mapping, calculates the probability that a QTL affecting the measured trait lies in an interval between two markers. Unlike interval mapping, CIM is able to produce a more refined output by analyzing additional markers outside the tested interval with multiple regression. The technique eliminates the effect of QTL that lie outside of the designated span between two markers. At every centimorgan, QTL cartographer calculates a likelihood ratio (LR) using the formula $2\log(L_0/L_1)$, where L_0 is the likelihood that there is no QTL within a given interval (the null hypothesis) and L_1 is the likelihood of the alternate hypothesis that there is a QTL in an interval. The higher the LR value, the higher the likelihood that there is a gene affecting the trait of interest within that region. One thousand permutations were performed (Churchill and Doerge 1994) to determine the significance threshold of $p \leq 0.05$. The effect size of each QTL peak was estimated by calculating the difference between the values of the phenotype for heterozygotes and for homozygotes under the peak LR value for each QTL and then scaling for the standard deviation of the phenotypic value. To calculate the position of a QTL the log of odds (LOD) output was used to create a LOD-1.5 interval which approximates 95% confidence intervals (Lander

and Botstein 1989). This is done by finding the maximum LOD score for each QTL as the likely location for a QTL, and then calculating the genomic location at which the LOD score drops by 1.5.

2.4 Results

Drosophila simulans and *D. mauritiana* backcross males (see Fig 2.1) who inherited a non-recombinant X chromosome had their sperm motility tested so that QTL mapping could be performed to find a genetic basis for the differences in fertility between individuals. The assays showed that no *D. simulans* backcross males had motile sperm, whereas approximately 15% of *D. mauritiana* backcross males (111 out of 760) had motile sperm (Fig 2.2). A single presumed *D. simulans* backcross male was found to have many motile sperm, but subsequent genotyping showed microsatellite alleles not present in the parental lines, which suggest that there was contamination in the cross, and so this individual was excluded from further analysis. As a procedural control, pure species males were tested for sperm motility as well. All *D. simulans* males (n=10) and nine out of ten *D. mauritiana* males had motile sperm, similar to the results of previous studies (Coyne 1985).

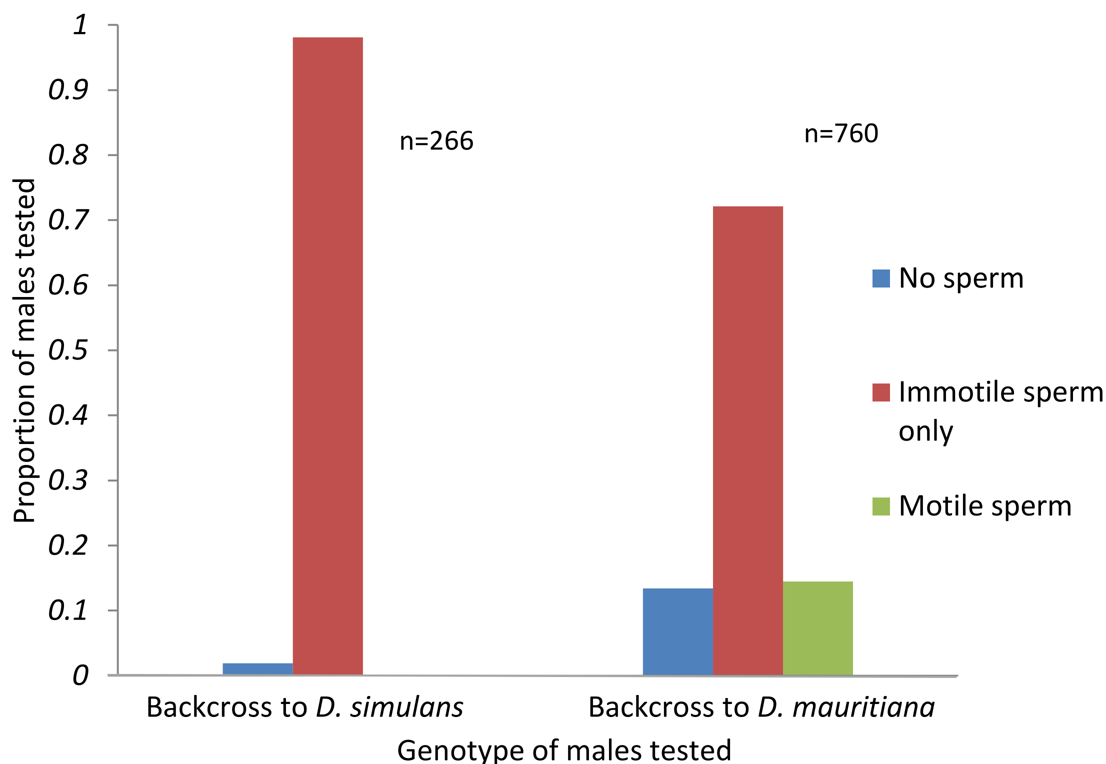

Figure 2.2 Proportion of backcross males with motile sperm and males with non-motile sperm. Backcross *D. simulans* (on left) and *D mauritiana* (on right) males, scored for presence or absence of sperm under dissection (see Methods). 'n' represents the number of individuals dissected, data for pure species males not shown.

As *D. simulans* backcross males were entirely sterile, it was not possible to perform QTL mapping to examine fertility as this analysis requires variation in the observed trait. The variation in *D. mauritiana* backcross male sperm motility was sufficient to analyze, so genotyping proceeded as planned. Genotyping of one 96-well plate of *D. mauritiana* backcross flies failed, likely due to DNA degradation, and so these samples were excluded from further analysis. Genotyping proceeded with the remaining 672 samples. Mapping was performed using multiple comparisons. The first comparison (shown in red in Fig 2.3) separated fertility scores into two categories: individuals with

motile sperm and individuals without motile sperm, independent of the presence of sperm in the latter category. The second comparison was similar to the first but included the presence of immotile sperm as an intermediate trait between sperm absence and motile sperm. The third comparison mapped QTL based on the presence or absence of sperm regardless of motility.

I identified six QTL that can account for the presence or absence of motile sperm (red line, Fig 2.3). The QTL account for 22% of the difference in phenotype (Table 2.2). Each QTL has a small to moderate effect, but none had an effect of less than 1% of the phenotype. When QTL mapping was performed with the inclusion of immotile sperm as an intermediate trait, the power of the analysis decreased as did the resolution, except with regards to the QTL in the middle of the second chromosome (green line, Fig 2.3). The R^2 values indicate that 17% of the phenotype can be accounted for by the identified QTL when examining both sperm presence and motility together. The overlap of the QTL identified by the two mapping methods would suggest that the different analyses identified the same genes. Most notable is the peak at 54 cM on the second chromosome which has a LOD score of 9.33 which is far higher than what is typical of hybrid sterility QTL mapping studies on autosomal loci (e.g. Moehring *et al*. 2006).

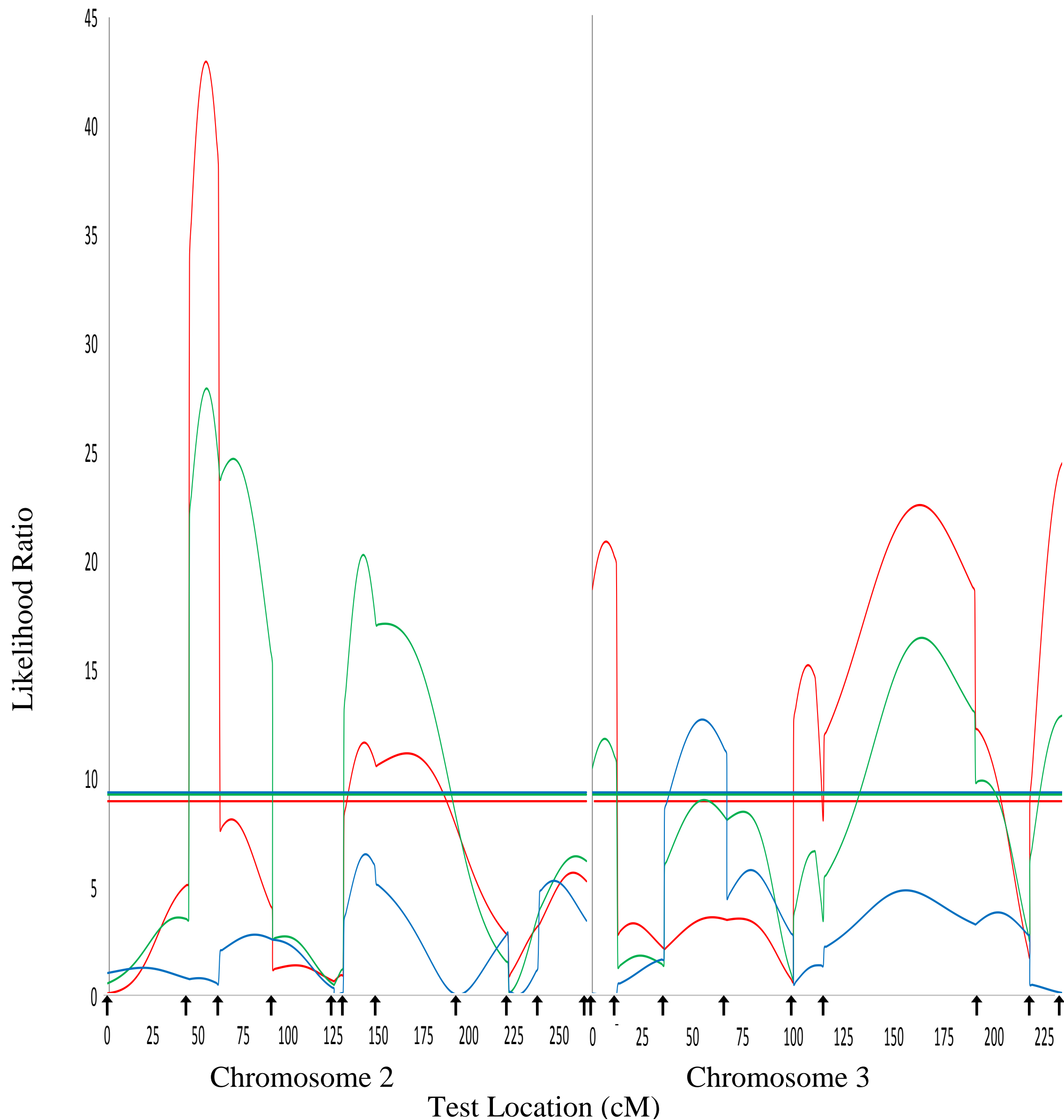

Figure 2.3 Composite interval map of *D. mauritiana* backcross male fertility. The second chromosome is on the left and the third chromosome on the right. Red represents a comparison of individuals based on presence or absence of motile sperm; green is the same, but also includes information on sperm presence or absence; blue represents the analysis based strictly on presence or absence of sperm. The corresponding horizontal lines show the significance thresholds for each trait. Arrows represent the locations of molecular markers used in genotyping.

Table 2.2 Hybrid sterility QTL locations and their effects. Position of QTL is from the left hand of each chromosome, LOD-1.5 ranges are used to approximate a 95% confidence interval of a QTL's true location (Lander and Botstein 1989).

Comparison	Chromosome	Position (cM)	Additive Effect	R^2	LOD	LOD-1.5 (cM)
Presence of motile sperm	2	54	0.19	0.064	9.33	46 -62
	2	142	0.1	0.017	2.51	131-208
	3	7	0.12	0.027	4.53	0-13
	3	107	0.1	0.02	3.3	100-115
	3	164	0.18	0.061	4.89	131-191
	3	234	0.13	0.03	5.31	224-234
Presence of sperm and sperm motility	2	53	0.23	0.044	6.02	45-83
	2	142	0.2	0.033	4.39	131-181
	3	6	0.14	0.017	2.55	0-13
	3	165	0.26	0.057	3.56	134-201
	3	234	0.15	0.016	2.78	218-234
Presence of sperm	2	55	0.11	0.028	2.76	36-67

The analysis obtained based only on the presence or absence of sperm yielded only one significant QTL (blue line, Fig 2.3). This peak contributes less than 3% of the phenotype. This information, taken in the context of the phenotypic effect data above, shows that this experimental protocol showed the greatest strength in accounting for motile sperm. The protocol lacks power in resolving variation in sperm presence and therefore provides less information about when in development sperm production is affected. Combine all the protocols are capable of describing seven QTL located throughout the autosomes.

An interesting phenomenon was observed among the progeny of the crosses: although attached-X F_1 females are homozygous for a recessive white eye trait on their X chromosome, approximately 15% (19 out of 125 examined) of interspecies F_1 females had red eyes. Moreover, these females were found, in a separate cross with both *D. simulans* and *D. mauritiana*, to be unable to produce offspring. The red eyed females are, therefore, likely to have both the maternal attached-X chromosome and a paternal X chromosome that lacked the recessive white eye mutation; the single functional copy of the *white* gene would cause these XXX females to have red eyes. The pure species attached-X stock did not show this phenotype, with 0 out of 96 individuals examined being red-eyed females.

2.5 Discussion

The discovery of autosomal loci in *D. simulans* and *D. mauritiana* that are capable of causing sterility when they are heterozygous is a unique result. The ability of the loci to produce sterility in the heterozygous state implies that the alleles they include are capable of acting dominantly and capable of contributing to sterility in F_1 individuals. As F_1

sterility is a greater barrier to interspecies gene flow than sterility in subsequent generations, these loci can have a greater contribution towards species isolation than loci that would act only when homozygous. In the last case, sterility would appear only in a fraction of individuals and not until the F_2.

All of the males from the *D. simulans* backcross lacked motile sperm; there are two possible reasons for this. The first is that there is an epistatic interaction between the *D. simulans* X chromosome and the *D. mauritiana* Y chromosome, an idea that has been previously proposed (Coyne 1985). The second is that sterility is caused by interactions between the X chromosome of *D. simulans* and the autosomes of *D. mauritiana.* For this second option to be true, there would need to be a large number of interactions capable of causing sterility. Variation exists in the genotypes of backcross males, and if sterility was caused by only a small number of X/autosome interactions, a portion of the 266 tested males would be free of such negative interactions due to the chance nature of recombination, and would be fertile.

The fertility seen in the backcross to *D. mauritiana* males is consistent with the Dobzhanksy-Muller model and the dominance theory. If one assumes that the QTL identified correspond to alleles, with the possibility of multiple sterility alleles at each locus, two types of interactions are capable of causing sterility in the observed backcrosses. The first is an interaction between a *D. simulans* dominant autosomal allele and a recessive allele on the *D. mauritiana* X chromosome. Such an interaction would be consistent with both the Dominance and Dobzhanksy-Muller theories. The second possible interaction would be between a dominant *D. simulans* autosomal gene and a *D. mauritiana* autosomal gene that may be recessive (and would be homozygous in some

individuals); this interaction is only be compatible with the Dobzhanksy-Muller model, which as previously mentioned states that hybrid dysfunction is caused by interactions at two (or more) loci. Evidence derived from other sources suggests that genetic interactions of the type proposed by either of these theories are not universal, with regards to speciation, even within the genus *Drosophila* (Masly *et al.* 2006).

Combining the results obtained with the three comparison methods I used, seven autosomal QTL have been found to contribute to hybrid sterility. Also of note is the observation that QTL do not appear to be clustered in any one specific region of the autosomes; however, as each QTL may represent multiple genes, the initial assessment may be an underestimate. Earlier mapping studies have found that X chromosome QTL have a larger effect and autosomal regions were only coarsely mapped (Coyne 1984; Cone et al 1991) Previous QTL mapping studies have found few autosomal loci, all of small effect. An example can be seen in crosses with *Drosophila santomea* and *D. yakuba*, QTL mapping discovered three and five QTL, according to which parental species F_1s were backcrossed, with only two of these QTL being located on the autosomes, and contributing less than 4% of the phenotypic variance (Moehring *et al.* 2006a). Chang and Noor (2007) identified four QTL that were capable of acting dominantly on hybrid sterility in *D. persimilis* and *D. pseudoobscura bogotana.* Slotman *et al.* (2004) provided an example of a rare study that was capable of finely mapping several dominantly acting loci in the mosquito species *Anopheles gambiae* and *A. arabiensis*.

It is interesting to note that none of the analysis methods are able to account for the majority of the variation seen. Possible reasons are firstly that many loci with small

effects may have gone undetected or secondly that a substantial amount of variation is non-heritable. A significant contribution of non-heritable factors is unlikely as a similar phenomenon is not seen in pure species individuals.

This study has identified fewer loci responsible for hybrid sterility than have previous studies. Introgression mapping of *D. simulans* and *D. mauritiana* identified 19 such QTL on the third chromosome alone (Tao *et al.* 2003). Another study gave similar results with a larger number of autosomal sterile factors, with a slightly greater abundance on the third chromosome (True *et al.* 1996). Even if one assumes that the third chromosome has more hybrid sterility loci compared to the second chromosome, this is far in excess of what I have resolved in the current study. Two likely reasons may be proposed to account for this discrepancy: first, my crossing schemes were designed to identify only loci that were capable of acting dominantly. The majority of genes involved in hybrid dysfunction may act recessively through loss of function mutations, as proposed by Orr (1993). Introgression mapping uses homozygous segments of DNA, and therefore, it cannot distinguish between genes that are recessive or dominant and so is likely to identify more genes in total. A second reason one would expect to find fewer loci through my method of mapping is that introgressions allows for improved resolution as small introgressed segments can be analyzed one at a time. Tightly linked genes would be counted separately in contrast with QTL mapping which may count linked genes as a part of one QTL; this is one of the weaknesses of QTL mapping. Rockman (2012) pointed out that the identification of a small number of major genes that contribute to a phenotype can be caused by a selection bias towards analysis methods capable of detecting large effect QTL. Large-effect QTL are not uninformative so long as the role of small-effect

additive QTL is not ignored. With each method of analysis used in this study, less than one fourth of the variation in phenotype can be explained. Therefore, it is likely that a large number of genes with small effects also contribute to sterility.

A large number of autosomal sterility loci have previously been identified by introgression mapping, but it is difficult to determine whether some of these genes are responsible for the QTL identified in my study. One would only expect there to be overlap between dominant genes however it is not possible to differentiate recessive/dominant loci using homozygous introgressions. By chance one would expect some introgressed segments capable of causing sterility to coincide with the QTL peaks. However, the regions corresponding to my QTL have not been singled out as being of interest (True *et al.* 1996; Tao *et al.* 2003). Previous studies with introgression mapping have looked at segments of *D. mauritiana* DNA in a *D. simulans* background (True *et al.* 1996; Tao *et al.* 2003); whereas, my study mapped *D. simulans* segments of DNA in a *D. mauritiana* background. Since there is no expectation that the same genes cause sterility when they originate from different species, one might not expect an overlap in the regions identified as causing sterility.

A mutation screen of *D. melanogaster* to identify the number of genes involved in male fertility estimated a minimum of 500 genes located throughout the genome (Wakimoto *et al.* 2004). This is far in excess of the number of loci identified as contributing to hybrid male sterility, either by my method or by introgression mapping. This would suggest that mutations capable of causing complete sterility in hybrids are rare, or that the rate at which these mutations arise is low. The observation that genes previously identified in hybrid sterility appear to be experiencing positive selection as

shown by increased rates of non-synonymous mutations would support the idea that most mutations have a low probability of causing dysfunction (Ting *et al.* 1998; Barbash *et al.* 2003).

The regions identified in this study are large with the largest region containing approximately 1700 known and predicted genes. It is, therefore, not possible to identify individual candidate genes from this study alone. The identification of single genes would require further recombination mapping to refine the regions to a smaller number of genes, or a different mapping technique that builds upon the information provided here. The ideal outcome of future research would be the characterization of candidate genes within each QTL, as well as an understanding of the molecular interactions between these genes or their gene products.

The question why F_1 hybrids appear to tolerate an extra X chromosome while pure *D. simulans* flies do not would undoubtedly benefit from further research. I can only speculate that the phenomenon may be caused by a lethal factor, possibly acting through a dosage effect that is lacking on the *D. mauritiana* X chromosome and present on *D. simulans* X chromosomes.

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Chapter 3

3 Lifespan depression in hybrids of *Drosophila simulans* and *D. mauritiana*

3.1 Abstract

Post-zygotic reproductive isolation in *Drosophila* has been the subject of intense research, especially with regards to hybrid sterility and inviability. This chapter examines a more subtle mechanism of species isolation - *i.e.*, reduction in lifespan. When performing crosses of attached-X *D. simulans* females (in which the two X chromosomes are fused together) with *D. mauritiana* males, I noticed that F_1 and backcross females had a reduced lifespan relative to pure species individuals. This study uncovered a reduction in the innate lifespan of hybrid females, as well as a reduction in their lifespan that can be attributed to a disproportionate effect of the cost of mating on the hybrids. The phenomenon was not observed in hybrids that lacked an attached-X chromosome. Hybrid dysfunction is thought to be caused by divergence at multiple loci, which would suggest that the genetic cause is an interaction between a recessive X-linked gene and a dominant autosomal gene. These data provide insight on a mode of reproductive isolation that is relatively poorly studied but nonetheless important. A decreased lifespan will act as a barrier to gene flow by reducing egg laying opportunities.

3.2 Introduction

When members of two species mate, the resultant offspring (if any result) often are not as fit as the parental species, due to hybrid dysfunction. Hybrid dysfunction, a source of post-zygotic isolation, has been a well-studied facet of speciation research in the last two decades. A great deal of work has been done on hybrid sterility and hybrid inviability, the two best-known types of post-zygotic isolation (for example see: Coyne and Orr 1989; Presgraves *et al.* 2003; Moehring *et al.* 2006). Sterility and inviability are usually easy to examine as they are binary in nature, *i.e.*, an individual can be either alive or dead, either fertile or sterile. Individuals with reduced fertility are less likely to be examined. Sterile and inviable individuals have a fitness of zero and pass on no genetic material; however, there are also cases where hybrids merely suffer from reduced fitness. Here, I examine *Drosophila simulans* and *D. mauritiana* hybrids, which are already known to experience male hybrid sterility, and determine whether or not there exists an additional mode of post-zygotic isolation in these species, namely, decreased hybrid longevity in females.

A hybrid individual with a lifespan less than that of either parent can be expected to constitute an incomplete barrier to gene flow between the two parental populations. All else being equal such a hybrid would be less fit than the parental species because it would not be able to mate as often, and therefore, would produce a smaller number of offspring. Any species pair that produces offspring with a shorter lifespan would suffer from a subtler and less complete form of post-zygotic isolation than those that produce sterile or inviable hybrids.

Another phenomenon that could reduce hybrid fitness is the cost of mating, which is a key feature of the mating arms race or sexual antagonism (Chapman *et al.* 1995). The

energy expended during mating and producing offspring can affect an individual's fitness. Mating often burdens the female with a higher cost than the male (Crudgington and Siva-Jothy 2000), who benefits if the female produces a large number of his own offspring. Females will in turn develop mechanisms that limit the harmful effects of mating. An example can be seen in ducks (*Anas platyrhynchos*), where males often force themselves upon females (Brennan *et al.* 2007). In turn, female ducks have developed a vaginal tract that resists insemination by means of blind ducts (dead ends) and corkscrew-shaped genitalia (Brennan *et al.* 2007). These structures allow the female to reproduce only with desirable and presumably fit males, as they make female cooperation necessary. The mating arms race can also be observed in individuals that practice traumatic insemination, such as bed bugs (*Cimex lectularius*), where the male sex organ is used to pierce the female (or hermaphrodite) in order to copulate, leading the females to develop methods to reduce the harm, such as a thickened cuticle (Morrow and Arnqvist 2003).

The cost of mating phenomenon is also observed in the genus *Drosophila*, where it can entail a reduction in the lifespan of a female when mated to a male (Fowler and Partridge 1989). It would make sense that the resource costs of producing a greater number of eggs could cause a decrease in lifespan, although that was found to not be the only contributing factor, as males who produced no sperm could still effect a decrease in longevity (Fowler and Partridge 1989). Males produce, in their accessory glands, seminal proteins called Acps, or accessory gland proteins (Chapman *et al.* 1995), most of which have functions such as increasing ovulation and decreasing a female's receptivity to re-mating (for a summary see Wolfner 2002). For example, Acp62F has been shown to

reduce female life expectancy in *D. melanogaster* (Lung *et al.* 2002). Conversely Acp62F improves male fitness partially through its processing of another seminal protein, ovulin, which in turn increases egg laying (Mueller *et al.* 2008). Acp62F is also thought to play a role in sperm competition, which would also be expected to increase male fitness if precedence could be given to the sperm of one individual (Fedorka *et al.* 2011).

Drosophila females would be expected to evolve a defense against Acp male seminal proteins (Rice 1996). Females that were repeatedly exposed to the Acps from a particular male population would experience a selective pressure that would favor the co-evolution of defenses against the male proteins. A study in *D. melanogaster* showed that when the two sexes were not allowed to co-evolve the cost of mating was higher (Rice 1996). This was done by removing females from one population, mating them to males of another population, and, over several generations, keeping only male offspring and mating them to females from the original population. One might expect that if a female fruit fly was mated to a male of another species, the cost of mating might be even greater due to the inability of the populations to co-evolve for an extended period. *D. simulans* females, when mated to *D. mauritiana* males, do not appear to have a greater cost of mating compared to pure species pairs (Price *et al.* 2001). However, it is possible that *D. simulans*/*D. mauritiana* hybrids are unable to form a defense against the Acps from either of the parental species due to epistatic interactions between the alleles of the female response genes of the different species. If hybrid females needed both copies of Acp response genes to be from the same species, then a hybrid would not have a complete defense.

To contribute to our understanding of this phenomenon, I quantified the decrease in lifespan which I had observed in *D. simulans*/*D. mauritiana* hybrid females which have an attached-X chromosome. I also analyzed the longevity of these hybrids when they were paired with males of the parental species, and compared their longevity to females who were raised in the absence of males, with the expectation that mating would reduce lifespan further.

3.3 Materials and methods

3.3.1 Stocks used

The *D. mauritiana* SYN, *D. simulans* Florida City (FC; Drosophila Species Stock Center), and attached-X *D. simulans* (C(1)RM w/1z^5; provided by D. Presgraves) stocks, as well as the F_1s and backcrosses, were kept on standard Bloomington medium (Bloomington *Drosophila* Stock Center) at 23°C and a 14:10 hour light:dark cycle. Attached-X hybrids were used because the reduced lifespan phenomenon was initially observed during experimentation on male siblings. Five-day-old virgin attached-X *D. simulans* females were crossed with 1-6 day old *D. mauritiana* males. To obtain backcrosses, virgin F_1 females were collected daily and immediately paired with either *D. simulans* FC or *D. mauritiana* males (Figure 3.1). Additional (standard) F_1s were generated by crossing *D. simulans* FC females to *D. mauritiana.* Table 3.1 provides a list of all of the groups tested.

3.3.2 Longevity assay

To test for innate reduction in lifespan, that is, a reduction in lifespan not caused by environmental factors, longevity assays were performed on hybrid females from both

directions of backcross as well as attached-X and ‘standard’ F_1s. F_1 males were also tested. Individuals were placed three to a vial. Every twelve hours, beginning at lights on, vials were examined to determine if any individuals had died by looking for movement after tapping the vial and probing the fly with a paintbrush. The dead flies were removed. Flies were transferred to fresh vials every two days, two hours before the evening examination. To test for a reduction in longevity due to mating, females from both *D. simulans* and *D. mauritiana* backcrosses, as well as both types of F_1, were also tested for longevity while paired with either *D. simulans* FC or *D. mauritiana* males. Pairing with males was used as a substitute to testing directly for mating, although a subset of vials were examined for larvae to ensure that mating occurred in all crosses. Three females were placed in a vial with five males. Longevity was measured with the same method as for unmated flies, with the addition that males were also removed from vials upon their death. The lifespans of all tested individuals were compared to the lifespans of pure species individuals from previous studies.

3.3.3 Statistical analysis

Survival data of the tested individuals were analyzed using multiple Mantel-Cox tests and compared to the controls, in which I examined the longevity of F_1 males and F_1 females without an attached-X. Comparisons were made between all individuals of the same mating condition (for example, those paired with *D. simulans* males), and all individuals of the same cross (for example, all F_1 females). Five comparisons for each mated group, and eight for each unmated group (table 3.1) were made and through Bonferroni corrections alpha-values of 0.01 (mated) and 0.00625 (unmated) were used as a significance threshold for each individual test.

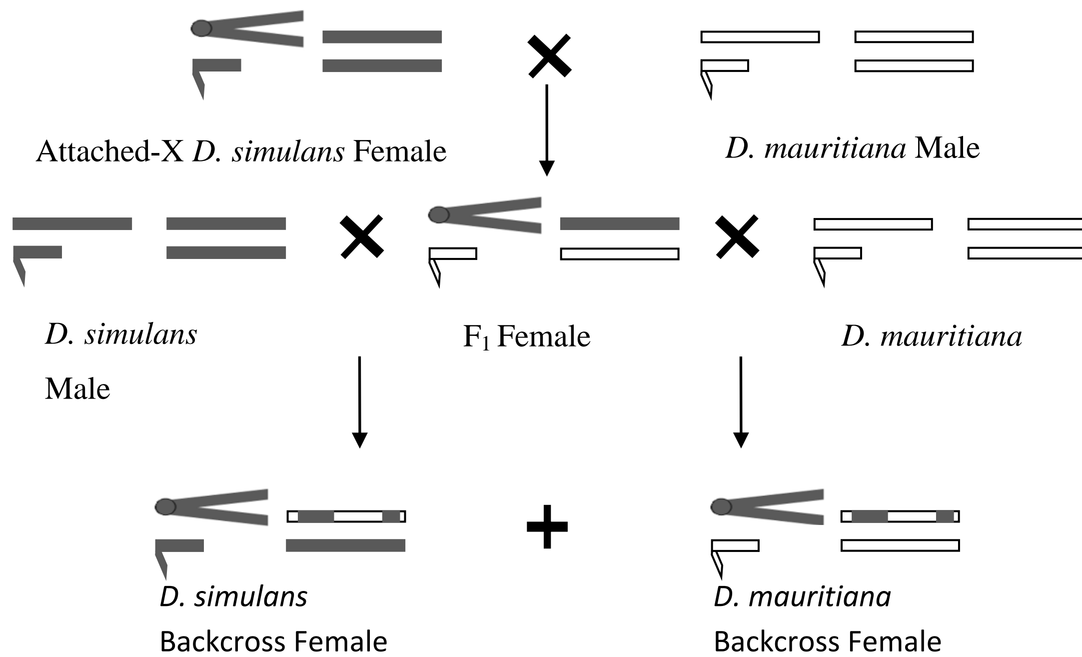

Figure 3.1 The crossing scheme used to obtain F_1 and backcross individuals with an attached-X chromosome. The autosomes (2nd 3rd and 4th chromosomes) are represented by a single pair of bars. The sex chromosomes are shown on the left for each individual with a short, hooked bar representing the Y chromosome and two bars joined together representing an attached-X chromosome. Colors represent the species of origin for the genetic material (grey for *D. simulans* and white for *D. mauritiana*) and bicolor bars represent recombinant chromosomes.

3.4 Results

3.4.1 Longevity of unmated flies

Table 3.1 and Figure 3.2 show the survival data for longevity assays of attached-X F_1 and backcross females. The data show that all of the experimental groups have reduced average lifespans ranging from 8.5 to 17.8 compared to the reported lifespans for pure species individuals which have and average lifespan between 40.85-47.45 days depending on the cross. The females resulting from the backcross to *D. mauritiana* had a significantly reduced lifespan relative to the *D. simulans* backcross and the F_1 ($p<0.001$ for both comparisons). Backcross *D. simulans* and F_1 females were not significantly different from each other in terms of lifespan ($p=0.260$). However, all of the female crosses (*D. simulans* and *D. mauritiana* backcross females and F_1s) had significantly shorter lifespans than F_1 males ($p<0.001$ for all comparisons). The backcross and F_1 population were all significantly different ($p<0.001$ for each comparison) from the unmated pure species lines with *D. mauritiana* surviving the longest with an average lifespan of 47.45 days flowed by the *D. simulans* FC and attached-X lines with average lifespans of 45.97 and 40.85 days respectively. This is slightly less that the literature value for *D. simulans* (~60 days) but this could be due to differences in the lines tested (Nikitin and Woodruff 1995). Also of interest in the difference in shape of the survivability curves between the pure species and experimental groups; The experimental groups have only moderate mortality early on which increases later in life, the inverse of what is seen in the other populations (Fig 3.2 F).

Table 3.1 Average lifespan of all the tested crosses as well as the numbers of individuals tested. A '*' denotes right-censored data; means of these two controls are underestimated since the assay was ended at day 35, when the majority of individuals were still alive. F_1 (FC) females act as a control as they lack an attached-X chromosome.

Test Group	Partner	Average Lifespan (days)	Individuals tested
D. simulans backcross females	Unpaired	15.3	196
D. simulans backcross females	*D. simulans* males	4.4	190
D. simulans backcross females	*D. mauritiana* males	7.7	175
D. mauritiana backcross females	Unpaired	8.5	337
D. mauritiana backcross females	*D. simulans* males	4.3	196
D. mauritiana backcross females	*D. mauritiana* males	3.7	174
F_1 females	Unpaired	17.8	62
F_1 females	*D. simulans* males	4.7	189
F_1 females	*D. mauritiana* males	4.9	147
F_1 males	Unpaired	59.4	64
F_1 (FC) females	*D. simulans* males	>31.1*	46
F_1 (FC) females	*D. mauritiana* males	>32.3*	60
D. simulans (FC) females	Unpaired	45.97	28
D. simulans (attached-X) females	Unpaired	40.85	27
D. mauritiana	Unpaired	47.45	31

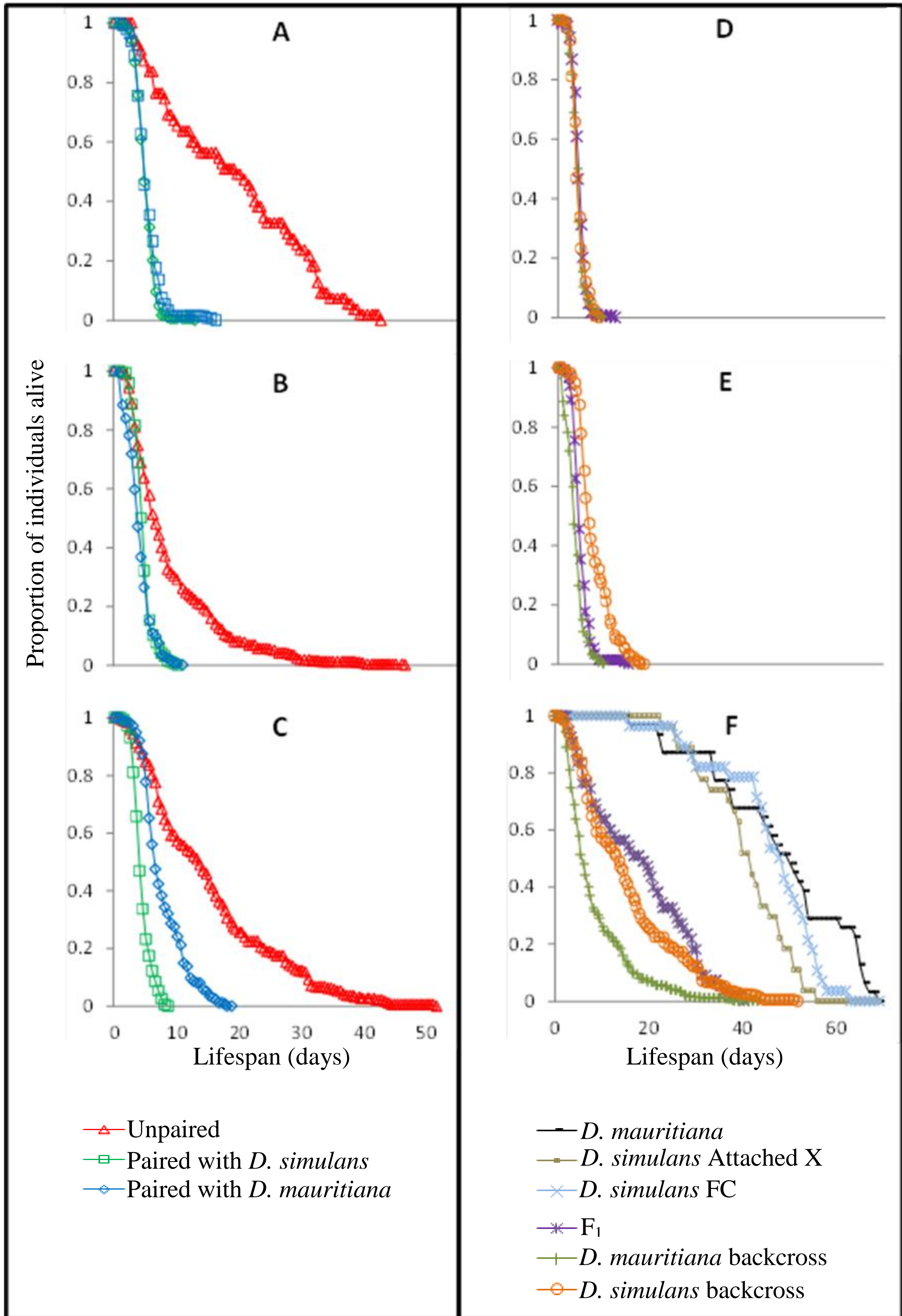
A
B
C
D
E
F
Proportion of individuals alive
Lifespan (days)
Lifespan (days)
Unpaired
Paired with D. simulans
Paired with D. mauritiana
D. mauritiana
D. simulans Attached X
D. simulans FC
F1
D. mauritiana backcross
D. simulans backcross

Figure 3.2 Survival curves for F_1 and backcross females both paired and unpaired. Data are represented as the proportion of individuals alive. The sample sizes are listed in in Table 3.1. Graphs A, B and C each represent a line of female fly and the different colors of curves are used to denote mating partners. Graph A shows F_1 individuals, B shows the *D. mauritiana* backcross population and C shows the *D. simulans* backcross population. Graphs D, E, and F use the same data, but are separated based on mating partner, with each color of curve representing a line of female used. Graph D shows the populations that were mated to *D. simulans* FC males, Graph E shows the individuals mated to *D. mauritiana* males, and graph F shows individuals that are unmated with the inclusion of pure species populations.

3.4.2 Longevity of paired flies

There was a significant reduction in lifespan of F_1, *D. simulans* backcross and *D. mauritiana* backcross females when they were unmated compared to when they were paired with males of either parental species and given the opportunity to mate ($p<0.001$ for each comparison). Vials from each set of crosses contained larvae, confirming that mating had occurred during cohabitation of males and females. In addition, a pattern was evident that backcross females mated to males of the same species as their fathers experienced a greater reduction in lifespan than when mated to the other pure species males. The trend was significant in *D. simulans* backcross females, which did not survive as long when paired with *D. simulans* males (average 4.4 days), as opposed to being paired with *D. mauritiana* males (7.7 days, $p<0.001$). A significant trend was not detected in the *D. mauritiana* backcross females, who showed a decreased average survivability when mated to *D. mauritiana* males (3.7 days) compared to being paired

with *D. simulans* males (4.3 days, p=0.038). Although this p value was low one must recall that a significance threshold was set at 0.01 to account for multiple comparisons. F_1 females, as well, did not show a large difference between their reductions in lifespan caused from mating with *D. simulans* males compared to mating with *D. mauritiana* males (p=0.067).

There also appeared to be more similarity in the lifespans of treatment groups that were mated to *D. simulans* males. The longevities were not significantly different between *D. simulans* backcross females and *D. mauritiana* backcross females when either were paired with *D. simulans* males (p=0.617). Neither of those groups were significantly different from F_1 females paired with *D. simulans* (p=0.220, p=0.015, respectively).

3.5 Discussion

The intrinsic lifespan of the attached-X F_1 and backcross females was reduced relative to pure species individuals. By intrinsic lifespan, I mean the reduction in lifespan that can be seen when females are not exposed to males. There is also a further reduction in lifespan caused by the increased cost of mating. An interesting result of this experiment is that the reduction in lifespan due to the cost of mating is more severe in the crosses presented here than the reduction initially observed in pure species *D. melanogaster*: we observed a mating-induced reduction in mean lifespan of 50-74% depending on the individual cross, while Chapman *et al.* (1995) reported an approximately 40% reduction in lifespan due to mating in *D. melanogaster*. This lifespan reduction caused by the increased cost of mating is by itself a barrier to gene flow, and therefore, is capable of contributing to species separation, as is the intrinsic reduction in lifespan. It is unknown if the reduction

in lifespan and the increased cost of mating share a single genetic basis or if the two phenomena are caused by inter-specific divergence in a single pathway.

A prevailing theory of the genetic basis of hybrid dysfunction is the Dobzhanksy-Muller model, which states that dysfunction is caused by interactions between alleles at two or more loci (Bateson 1909; Dobzhansky 1934; Muller 1939). If an ancestral species with genotype *AABB* has diverged in one population to *AAbb*, and in a second population to *aaBB*, the possibility exists for a dysfunctional interaction between these two genes if a hybrid with genotype *AaBb* is created. This is because the newly evolved *a* and *b* alleles have not co-evolved together, such that the fitness-reducing effects of their interaction have not been selected against. This model is usually mentioned with regards to sterility or inviability, but it is equally plausible as an explanation for decreased lifespan.

A decrease in lifespan relative to pure species individuals is not seen in F_1s that lack an attached-X chromosome. Figure 3.3 shows the genetic interactions that could cause the dysfunction seen in the attached-X hybrids. The presence of two X chromosomes from one species would allow an interaction between a recessive gene on the X chromosome with a gene on the autosomes or Y chromosome. Note that here the term recessive refers only to effect of the gene on hybrid dysfunction and makes no statement as to the gene's normal function. An interaction between the X chromosome and an autosome is the most relevant in the context of species separation as it is only certain crosses that would allow a Y chromosome to be present in a female. The X/autosome interaction is also the most likely cause of a gene-based interaction because the Y chromosome of these *Drosophila* species, while large, contains only small number of genes.

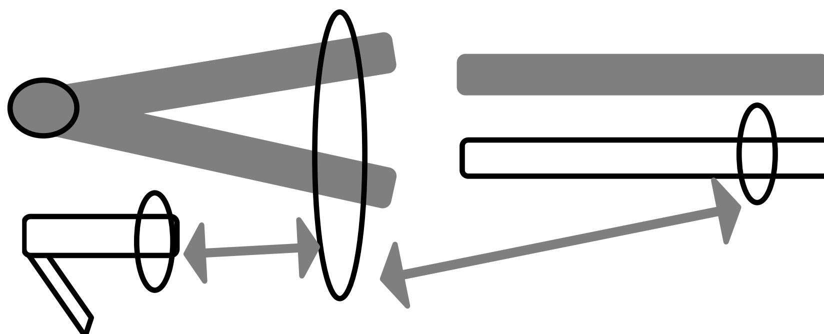

Figure 3.3 The possible gene interactions that could cause a decrease in hybrid lifespan. One interaction is between loci on the attached-X chromosome and the Y chromosome. The other is an interaction between loci on the X chromosome and the autosomes.

Figure 3.2 shows that none of the mated backcross females managed to achieve a normal lifespan or even a lifespan approaching that of their unmated counterparts. Some individuals with normal lifespans would be expected if the genetic basis of this trait were the result of a single X/autosome interaction, as approximately 50% of the females from the *D. simulans* backcross would be expected to be homozygous for *D. simulans* alleles at each particular locus. The idea of an X/autosome interaction as a cause for decreased lifespan is still viable when one considers the possibility that a large number of genes on the autosomes may be contributing. This could seem unlikely until one realizes that more than 60 autosomal genes contribute to hybrid sterility between *D. simulans* and *D. mauritiana* (True *et al.* 1996).

If the reduction in lifespan in unmated females is the result of a separate genetic interaction from that of the cost of mating one must ask why males are seemingly unaffected by reduced lifespan. Hybrid sterility is thought to have a different genetic basis in males and females (Coyne 1985; Orr 1987; Orr 1987) whereas inviability is

thought to be caused by the same genes in both sexes (Orr 1993; for a review see Coyne and Orr 2004). This makes sense because the pathways responsible for fertility are quite divergent between the sexes, which is not the case with viability. Although it would seem reasonable that genes for longevity also affect both sexes, this has been shown not always to be the case. The gene *superoxide dismutase* (*SOD*) significantly increases female life expectancy in *D. melanogaster* individuals with a variety of genetic backgrounds but *SOD* only affects males of a few genetic backgrounds (Spencer *et al.* 2003). Conversely the gene *methuselah* reliably increases male longevity but only increases female longevity at certain temperatures (Mockett and Sohal 2006). The other possibility for why the reduction in lifespan is limited to females is that the males in the F_1 inherit their X chromosome from *D. mauritiana* and consequently would possess different alleles of the genes that cause a decrease in lifespan.

Future studies should aim at mapping of the genes responsible for both the decrease in lifespan and the cost of mating. These genes could explain the variation seen in the lifespan of backcross individuals. By examining the F_1 individuals it is easy to see there is already much variation in individuals that share the same genotype, and therefore one could predict that much of the variation in backcross individuals in non-heritable.

As mentioned above, the *D. melanogaster* protein Acp62F has been shown to be at least partially responsible for the cost of mating in the genus *Drosophila*. It would seem as though the most likely candidate for the molecular cause of the cost of mating seen in the flies examined in this study would be the female targets of this protein. Acp62F encodes a protease inhibitor and has been shown to decrease the activity of trypsin (Lung *et al.* 2002). The majority of Acp62F localizes to the female reproductive

tract but approximately 10% is absorbed into the hemolymph where it can affect other organs (Lung *et al.* 2002). When the protein is present throughout the female's body it is toxic (Lung *et al.* 2002). This is also the case for three other less well characterized ACPs (Acp70A, CG8137, and CG10433; Mueller *et al.* 2007). Acp62F as a protease inhibitor is thought to interfere with essential protease cascades in females (Lung *et al.* 2002). The specific pathway that these proteins disrupt has not been identified but it is reasonable to predict that female variation in this pathway could be responsible for variation in the cost of mating shown here.

Lifespan reduction and increased cost of mating may be common to hybrids of several species pairs. Attached-X females allow for F_1s that are homozygous for genes on the X chromosome. This would not be seen in most interspecies F_1s as they lack an attached-X chromosome. Later generations of hybrids such as F_2s and backcrosses will have some individuals that are homozygous for each locus on the X chromosome thus also exposing recessive genes. Why is lifespan reduction not commonly observed in later generations of hybrids? If it is common in the tested genus that many individuals die at a young age, even in pure species populations, it is possible that individuals affected by hybrid lifespan reduction do not make up a large enough portion of the total population to create an easily observed phenotype. In other words enough individuals would need to be affected by this form of hybrid dysfunction that their deaths were not masked by already existing mortality.

The experiment described in this chapter has yielded observations about an as of yet understudied mode of post-zygotic isolation, and raises the question of how common lifespan depression is. It is quite possible that this phenomenon is not unique to

Drosophila and in fact common among a variety of species including those already studied in speciation. This feature may not have been detected due to the subtle, non-binary nature of the trait or due to the possibility that lifespan depression is common in backcrosses as opposed to F_1s. Although decreased lifespan does not contribute to species separation as much as sterility or inviability it could still be a major contributor to speciation in species that do not suffer from another form of post-zygotic isolation.

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Chapter 4

4 Conclusions

In Chapter 2 I examined the causes of hybrid sterility in *D. simulans* and *D. mauritiana* using a crossing scheme involving females with an attached-X chromosome. This allowed for the creation of backcrossed individuals that had a recombinant set of autosomes in the background of non-recombinant sex chromosomes. The backcrosses increased the sensitivity of testing for the effect of autosomal loci because the effect of the X chromosome remains constant. The effect of the X chromosome is useful to overcome, as there is a large likelihood that speciation genes will map to the X chromosome and also individual genes on the X chromosome have a large effect on the phenotype. Eliminating this effect may unmask some autosomal QTL that could not otherwise be detected. This study succeeded in identifying seven regions on the autosomes that contribute to hybrid sterility and are capable of acting in the heterozygous condition. The regions were only identified in *D. mauritiana* backcross hybrids, as *D. simulans* backcrosses produced no fertile males and therefore could not be subjected to QTL mapping.

While conducting the experiments reported in Chapter 2 I noticed that the female siblings of the hybrid males I created had a decreased lifespan and I examined this further to confirm and quantify my initial observations. The second set of experiments indicated that the lifespan of hybrid females was reduced by more than half, depending on the cross. In addition to the innate reduction in lifespan, I also quantified a decrease in

lifespan associated with the cost of mating. Hybrid females that were housed with males of both parental species have a lifespan that is even lower than that of their unmated siblings. The effect of mating reduced the lifespan of these females by approximately 50 to 75% depending on the cross as well as the species of the mate. This effect was only noticed in the hybrids that had an attached-X chromosome. When the experiments were repeated with F_1s from conventional detached X stock, there was no noticeable reduction in lifespan.

The Dominance Theory has been disproved as a universal explanation to Haldane's rule (Coyne 1985), although the mechanism proposed by the theory, interactions between recessive X chromosome genes and dominant autosomal genes, is still viable. The data presented in Chapter 2, namely the identification of autosomal genes that are capable of causing sterility when they are homozygous, gives credence to this theory. Chapter 3 also reports evidence of hybrid dysfunction that can be explained by a recessive X - dominant autosome interaction. The reduction in lifespan is only observed in females and therefore this experiment is inconsistent with Haldane's rule, which, as stated above, states that when only one sex is affected by hybrid dysfunction it will most likely be the heterogametic sex.

Another outcome of this thesis (Chapters 2 and 3) is the possibility that hybrid dysfunction is caused by interactions between a recessive X chromosome locus and a Y chromosome locus. The males obtained from the backcross to *D. simulans* (Chapter 2) could be sterile due to interactions between one or more loci on the X chromosome and many autosomal loci, or because of an interaction between loci on the X chromosome and on the Y chromosome. In Chapter 3 the decrease in lifespan could be caused by an

interaction between a locus on the attached-X chromosome and the Y chromosome, which these females possess, despite the fact that it is atypical for a female to have a Y chromosome. Although post-zygotic isolation can only be documented in some species pairs, it is worthy of study as it affects individuals that are at a more advanced stage of the speciation process (Coyne and Orr 1997).

This thesis has shed light on the genetic basis of one type of hybrid dysfunction that has already been well studied, *i.e.* hybrid sterility. Autosomal sterility loci have yet to be identified that are capable of explaining F_1 sterility in *D. simulans* and *D. mauritiana*. In addition this thesis describes a type of hybrid dysfunction that was not known to affect this species pair. Moreover, the study of the cost of mating between species has been rather limited and previous examination of the cost of mating between species has not shown the cost to be so severe (Price *et al.* 2001). This thesis provides a great deal of insight into the post-zygotic isolation of one of the most heavily studied species pairs.

Future research on the identity of the loci discovered in Chapter 2 or inferred in Chapter 3 will give further insight on the processes of hybrid sterility and decreased hybrid lifespan. The characterization of individual genes along with information on genes that have already been identified in connection to post-zygotic isolation should further clarify hybrid dysfunction with respect to the classes of proteins involved and the selection pressures, but with more emphasis on the genes of interest.

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