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# Cellular/Molecular Analysis of Interspecies Sterile Male Hybrids In Drosophila

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## Recommended Citation

# Abstract

Over time, genetic differences can accumulate between populations that are geographically separated. This genetic divergence can lead to the evolution of reproductive isolating mechanisms that reduce gene flow between the populations and, upon secondary contact, result in distinct species. The process of speciation is, thus, what accounts for the multitude of species that contribute to the rich biodiversity on Earth. Interspecies hybrid sterility is a postzygotic isolating mechanism that affects the development of hybrids, rendering them sterile. A notable trend, known as Haldane's rule, describes how heterogametic individual hybrids (e.g. males in *Drosophila*) are more susceptible to sterility than homogametic hybrids. My objective was to describe the stage at which spermatogenesis fails in hybrids produced from three interspecies crosses in *Drosophila.* Identification of the stage of spermatogenic failure may inform the underlying basis of Haldane's rule. I found that chromosomes do not separate after meiosis I, leading to non-disjunction, and the formation of needle-eye sperm that is immotile but not dead. Secondly, sperm head length is aberrant in aged (6 days) sterile hybrid males, suggesting improper nuclear packaging, even with bi-allelic expression of sperm protamines. Third, individual sperm nuclei possess two sperm tails, with two undifferentiated, but active, mitochondria. Finally, I mapped for genetic factors that contribute to the formation of needle-eye sperm and identified possible candidate genes. Together, these studies highlight that spermatogenesis fails at a consistent stage in sterile hybrid males in *Drosophila*, leading to the formation of paired sperm that are unable to fertilize. My findings suggest that the genetic basis of hybrid sterility may be universal within the genus *Drosophila*.

Keywords

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*This thesis is dedicated to the memory of John Vincent. We really started something awesome.*

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| | |
|---|---|
| BCS | backcross to *D. simulans* |
| BCM | backcross to *D. mauritiana* |
| BCS10 | backcross to *D. simulans* for 10 generations |
| BCM10 | backcross to *D. mauritiana* for 10 generations |
| BDM | Bateson-Dobzhansky-Muller model |
| DAPI | 4',6-diamidino-2-phenylindole |
| DSB | double stranded break |
| GFP | green fluorescent protein |
| HR | Haldane's rule |
| mauGFP | *D. mauritiana* GFP |
| mtDNA | mitochondrial DNA |
| NGS | next generation sequencing |
| PI | propidium iodide |
| protA | protamine A |
| protB | protamine B |
| PSIseq | phenotype-based selection and introgression followed by whole-genome resequencing |
| RFP | red fluorescent protein |
| simGFP | *D. simulans* GFP |
| TE | transposable element |
| TEM | transmission electron microscopy |

# Chapter 1

# 1    General introduction

Over evolutionary time, genetic differences can accumulate between populations that are geographically separated and that, as a consequence, do not frequently interbreed. This reproductive isolation and lack of gene flow between the potentially interbreeding populations can, through selection and drift, lead to genetic divergence and eventual speciation. This allopatric process of speciation likely accounts for a majority of taxonomic diversity on Earth.

## 1.1    Postzygotic isolation

Reproductive barriers that reduce the fitness of offspring produced from an interspecific (between species) mating event are described as "postzygotic barriers". Populations that have diverged along separate evolutionary paths can accumulate differences that are beneficial or neutral within each population. However, when these accumulated changes come together in $F_1$ hybrid genotypes, the novel allelic combinations and resultant phenotypes are exposed to natural selection for the first time. Because the hybrid genotypes have not evolved under selection per se they may prove to be dysfunctional or unfit. Postzygotic barriers are further classified into two broad categories. First, extrinsic postzygotic barriers describes hybrids exhibiting intermediate phenotypes that are not compatible with either parental niche or mating behaviour. In contrast, intrinsic postzygotic isolation describes incompatible genetic interactions that render the hybrid unfit (Coyne and Orr, 2004; Calhoun et al., 2016).

### 1.1.1    Extrinsic postzygotic isolation

Divergent selection on populations that inhabit different environments will lead to the fixation of advantageous alleles within each population that enhance their fitness within their respective environments (Schluter, 2000). As a result, extrinsic postzygotic barriers may evolve that cause hybrids exhibiting intermediate phenotypes to have reduced fitness within either parental niche (Rice and Hostert, 1993; Rundle and Whitlock, 2001; Coyne and Orr, 2004).This reduced fitness results from the interaction between the hybrid

phenotype and its environment (Rice and Hostert, 1993; Schluter, 1996a; Schluter, 1996b). Divergent natural selection on populations towards different fitness optima is a key component for sympatric speciation (Schluter 2000) and has been demonstrated in nature (e.g., Craig et al., 1997; Via et al., 2000; Naisbit et al., 2001).

One of the most notable examples of extrinsic postzygotic isolation is that of the hybrids formed between limnetic and benthic species of the stickleback fish *Gasterosteus aculeatus* (Hatfield and Schluter, 1999). Each species' morph is morphologically and ecologically differentiated and exhibits strong isolation that prevents the formation of hybrid offspring (i.e., prezygotic isolation) from the alternate morph (Hatfield and Schluter, 1996; Nagel and Schluter, 1998). Hybrids have intermediate phenotypes and demonstrate no reduction in fitness within a standard laboratory setting. Upon integration into either parental niche, however, hybrid fitness is reduced (Hatfield and Schluter, 1999). Thus, intrinsic postzygotic barriers from genetic incompatibilities are not the cause for hybrid dysfunction in this group, but rather an extrinsic barrier due to hybrids being poorly suited to either parental niche.

In addition to the above stickleback example, there are other well-documented studies of extrinsic postzygotic isolation. Sympatric species of pea aphids, *Acyrthosiphon pisum*, have adapted on different food sources, and produce $F_1$ hybrids with lower fitness when placed in either parental niche (Via et al., 2000). Gall forming tephritid flies (*Eurosta solidaginis*) that inhabit two different goldenrod hosts (*Solidago altissima* and *S. gigantea*) also produce hybrids that exhibit lowered survival rates compared to either parental species, a situation not due to genetic incompatibilities within hybrids (Craig et al., 1997). Divergent sexual selection on adult colour pattern preference in *Heliconius* sympatric species has also contributed to the evolution of extrinsic postzygotic barriers (Naisbit et al., 2001). Both species (*H. cydno* and *H. melpomene*) overlap in geographical region but exhibit strong assortative mating and low hybridization (Naisbit et al., 2001). Any $F_1$ hybrids produced possess intermediate phenotypes and are discriminated against by both parental species, but not discriminated against by other $F_1$ hybrids (Naisbit et al., 2001). In this instance, hybrids have lower fitness due to reduced attractiveness instead of a phenotype-environment interaction.

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## 1.1.2    Intrinsic postzygotic isolation

Intrinsic postzygotic barriers involve genetic incompatibles that affect hybrid fitness, often independent of environment (Coyne and Orr, 2004). Genetic interactions between parental genomes within a hybrid result in a developmental defect that renders the hybrid sterile (hybrid sterility) or the hybrid dies before sexual maturation (hybrid inviability).

### 1.1.2.1    Hybrid inviability

Interaction of two (or more) divergent loci, derived from two species, may interfere with the development of a hybrid before it reaches sexual maturation. Often, the hybrid dies during early stages of development and never matures into an adult (Coyne and Orr, 2004). While there are many examples of hybrid inviability, a historical and highly-studied example derives from *Drosophila melanogaster* mated to its sister species, *D. simulans* (Sturtevant, 1920). Female *D. melanogaster* crossed with male *D. simulans* generates viable daughters and sons that die during pupation. The reciprocal cross produces viable sons and daughters that die as embryos (Sturtevant, 1920). The genetic basis to this asymmetrical presence of female or male hybrid inviability has been well-studied, and the individual causal genes have been identified (Watanabe, 1979; Hutter and Asburner, 1987; Hutter et al., 1990; Sawamura et al., 1993; Sawamura et al., 1993; Sawamura et al., 1997; Presgraves et al., 2003; Tang and Presgraves, 2009). Since hybrid inviability was not the focus of my dissertation, I will not provide a thorough review of hybrid inviability, rather, I will briefly outline some important points on the genetic and cellular basis of hybrid inviability.

One prediction about the genetic basis of hybrid inviability is that the genes causing inviability can do so in both sexes. This was demonstrated in a classical experiment involving the use of a unique genetic tool found in *Drosophila,* wherein females of a particular strain possess two attached X chromosomes (Orr, 1993). Here, the physically linked X chromosome will be passed along to the next generation as an attached pair. Since lethality only affected male hybrids produced from the cross of female *D. melanogaster* with male *D. simulans*, this particular lethality was predicted to be caused by negative genetic interactions of the autosomes with recessive loci on the *D.*

*melanogaster* X chromosome. If the "attached-X" *D. melanogaster* females are mated with *D. simulans* males, the hybrid daughters will carry both X chromosomes from their mother, meaning that all X-linked loci come from *D. melanogaster*, just as they do in hybrid males. In this instance, hybrid "attached-X" daughters exhibit hybrid inviability (Orr, 1993). Furthermore, introgressed genomic regions from one species (that harbour genes for hybrid inviability) within the genetic background of another species, causes lethality in both hybrid sexes (Wu and Davis, 1993; True et al., 1996). Finally, hybrid inviability appears to be involved with maternally expressed genes, thus affecting males and females (Hutter, 1997). All of these results provide support for the theory that the same locus can induce lethality in both sexes.

Hybrid inviability can also involve incompatible maternal cytoplasmic transfer into the hybrid offspring. This includes instances of cytoplasmic incompatibility that will be discussed further below. For example, in cases where interspecific mating events lead to the production of only hybrid female offspring in *Drosophila* (i.e., female hybrid inviability)*,* it only occurs in one direction of the cross (Wu and Davis, 1993; Turelli and Orr, 2000; Coyne and Orr, 2004), suggesting certain females possess cytoplasm that are incompatible with male cytoplasm from a different species. Incompatible mitochondria appear to play an important role in hybrid inviability. In copepods, *Tigriopus californicus,* cytochrome *c* derived from the mitochondria of one population functions poorly when interacting with cytochrome *c* derived from the nuclear genome of another population (Burton, 1990; Edmands and Burton, 1999; Rawson and Burton, 2002).

## 1.1.2.2    Hybrid sterility

The production of hybrids from an interspecific mating event may not result in the individual dying before sexual maturation, as seen in hybrid inviability. Rather, hybrids may possess a developmental defect that negatively affects the reproductive system of the hybrid, rendering it sterile. The following section will outline three topics germane to hybrid sterility - i) the global trends of hybrid sterility, ii) theoretical models proposed, and iii) the genetic and cellular basis of hybrid sterility.

### 1.1.3    Trends for postzygotic isolation

### 1.1.3.1    Hybrid sterility evolves before hybrid inviability

It was originally proposed that hybrid sterility evolved at similar rates as hybrid inviability, based on a compilation of *Drosophila* interspecific hybrid data (Coyne and Orr, 1989). However, that hypothesis was refuted using a more sensitive analysis. Mathematical modeling predicts that hybrid sterility evolves more rapidly than hybrid inviability (Wu, 1992). This model is supported with data sets from *Drosophila* (Bock, 1984; Coyne and Orr, 1997) and mammals (Gray, 1954). In *Drosophila,* Bock (1984) noticed 199 cases of hybrid sterility and only 14 cases of hybrid inviability. In Gray's (1954) assessment of interspecific hybrids in mammals, 25 cases of hybrid sterility was observed, whereas no case of hybrid inviability was found. Furthermore, individual genes from one species that have been transgenetically introduced into the genetic background of another species yields more instances of male sterility than male inviability (Wu et al., 1992).

### 1.1.3.2    Haldane's rule

A well-noted phenomenon, known as Haldane's rule, has been observed in almost all interspecific hybrids. Haldane observed a trend that, if only one hybrid sex is inviable or sterile, individuals of the heterogametic sex (e.g. XY males or ZW females) are more likely to be affected than those of the homogametic sex (e.g. XX females or ZZ males; Haldane, 1922).

## 1.2    Theoretical basis of hybrid sterility and Haldane's rule

### 1.2.1    Dominance theory

The prevailing model to explain the genetic basis of hybrid sterility and Haldane's rule is the Dominance theory (Bateson, 1909; Dobzhansky, 1936; Muller, 1940; Orr and Turelli, 1995). This model describes the possible genomic differences between species that, when brought together in an individual, would be incompatible (Bateson, 1909; Dobzhansky, 1936; Muller, 1940), and render the hybrid offspring sterile or inviable. The core of this model, called the Bateson-Dobzhansky-Müller incompatibility model (aka BDM model),

explains how a pair of interacting loci can functionally diverge within two populations. If so hybrid sterility or inviability may be a result of genic incompatibility (Bateson, 1909; Dobzhansky, 1936; Muller, 1940). Orr and Turelli (1995) elaborated that hybrid sterility/inviability is a byproduct of genic incompatibility between one or more recessive X-linked factors of one species, that are interacting with a dominant autosomal-linked factor of another species. The recessive nature of the X-linked factor underlies Haldane's rule, since the genetic factor would only be expressed in heterogametic individuals. This genetic interaction would have a detrimental effect on the development of the hybrid offspring, such that the heterogametic individual would be sterile or inviable (Figure 1.1). These genic incompatibilities between species continue to accumulate even after speciation has occurred (Orr 1995; Matute et al., 2010), making it difficult to discern what incompatibilities underlie speciation *vs*. which arose after speciation. Genetic mapping studies have identified multiple genes influencing hybrid sterility. Most of these genes, however, do not meet the predictions of the dominance model (Perez et al., 1993; Perez and Wu, 1995; Presgraves et al., 2003; Masly et al., 2009; Tang and Presgraves, 2009).
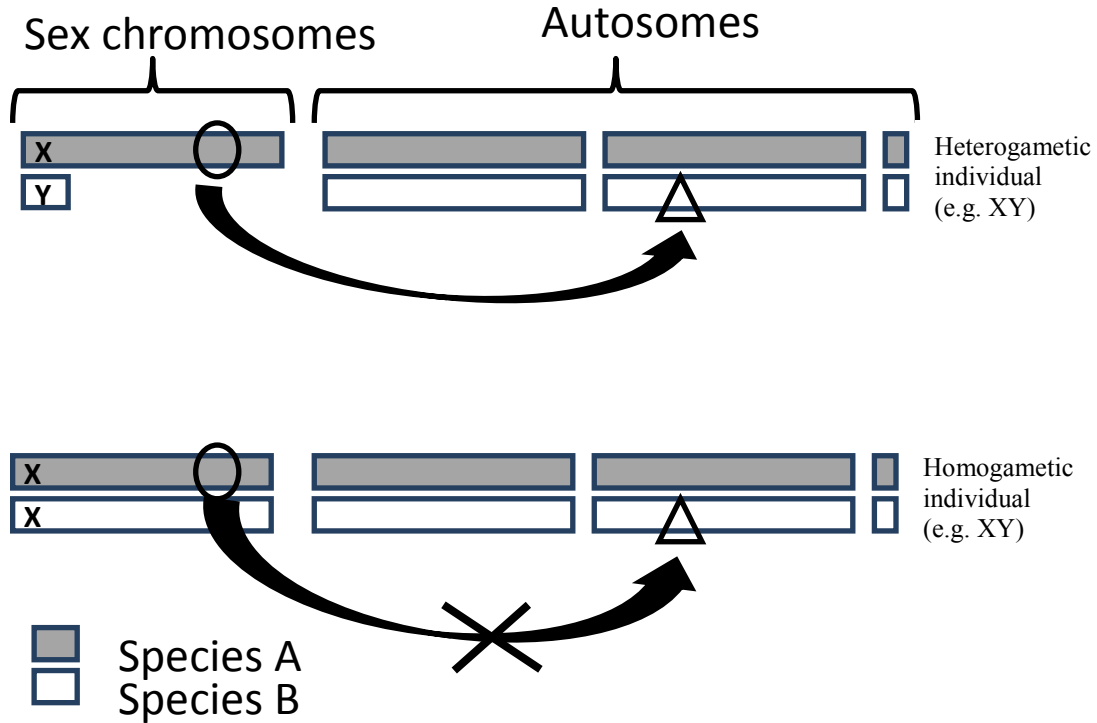
Figure 1.1: A pictorial representation of Haldane's Rule and the Dominance Theory in *Drosophila*. In a hybrid heterogametic individual (e.g. XY; homologous chromosomes are represented by horizontal bars), the hemizygous X chromosome possesses a recessive factor (circle) that was inherited from parental species A. This factor is unable to interact epistatically with a dominant factor on an autosome (triangle) that was inherited from parental species B. This interaction affects a vital developmental process, rendering the individual sterile. In contrast, a homogametic individual (e.g. XX) does not experience the same effect as X chromosome derived from species A is masked by the X chromosome derived from species B. As such, the recessive X factor inherited from parental species B is able to effectively interact with the autosomal factor, thus rendering the individual fertile.

## 1.2.2    "Faster-" evolution

Additional theories have been proposed to relate Haldane's Rule to the evolution of hybrid male sterility. Below are two theories that describes how hybrid males are more susceptible to sterility than hybrid females and how the X chromosome appears to play a much larger role in hybrid sterility.

## 1.2.2.1    Faster-male

Since hybrid individuals of the heterogametic sex are more likely to be sterile or inviable (Haldane, 1922), it's possible that loci involved in male reproduction are evolving faster than genes involved in female reproduction in those species where males are the heterogametic sex (Wu and Davis, 1993). The higher rate of evolution for male genes compared to female genes may be a by-product of sexual selection, as genes for attractiveness are more important in males than females (Wu and Davis, 1993). Alternatively, the process of spermatogenesis may be more susceptible to aberration within a hybrid, causing hybrid male sterility to occur more often than hybrid female steriliy (Wu and Davis, 1993). To test the "faster-male" theory within *Drosophila*, True et al. (1996) introgressed (crossed) pieces of *D. mauritiana* in the background of *D. simulans* and screened for sterile males and sterile females. Indeed, more sterile males were present than sterile females (True et al., 1996), an observation confirmed by subsequent studies (Hollocher and Wu, 1996; Tao and Hartl, 2003). Furthermore, male-specific genes are more likely to be mis-expressed in hybrids than female-specific gene, a pattern found in other interspecific pairings within the genus *Drosophila* (Reiland and Noor, 2002; Michalak and Noor, 2003; Ranz et al., 2004).

Testing of the "faster-male" theory in mosquitoes has provided additional support (Presgraves and Orr, 1998). For example, males from the *Aedes* genus do not have a degenerate Y chromosome, and possess genetically and morphologically indistinguishable sex chromosomes (Bhalla and Craig, 1970). Thus, males do not have a hemizygous X chromosome. Since males in this group have a transcriptionally-active Y chromosome, dominance alone cannot cause hybrid male sterility, but the "faster-male" theory can. As predicted by Presgraves and Orr (1998), sterile hybrids produced within

the *Aedes* genus do conform to Haldane's rule (Haldane, 1922), lending strong support to the "faster-male" theory.

Two limitations to the "faster-male" theory are: 1) the specific targeting of species where the heterogametic sex is male, and 2) lack of support for this theory for hybrid inviability (Coyne and Orr, 2004). Although the "faster-male" theory may be plausible in heterogametic XY hybrids (males), the theory does not account for heterogametic hybrid females, such as seen in *Lepidoptera* and birds. Further, it cannot be utilized as a general model for hybrid sterility.

### 1.2.2.2   Faster-X

The "faster-X" theory predicts that loci on the sex chromosomes, specifically the X chromosome, are evolving more rapidly compared to loci on autosomes (Charlesworth et al., 1987). If natural selection acts on favourable X-linked substitutions, these mutations will become fixed within a population faster than autosomal substitutions (Charlesworth et al., 1987). In *Drosophila,* it was noted that X-linked substitutions do have a greater impact on hybrid sterility than autosomal substitutions (Coyne and Orr, 1989). Additional studies have, however, generated mixed support for the theory (Coyne and Orr, 2004). Furthermore, the "faster-X" theory does not solely account for Haldane's rule, as both sexes should be affected (Coyne and Orr, 2004). As such, modifications to the predictions for the "faster-X" theory will link the large X-effect to either the Dominance theory or "faster-male" theory.

### 1.2.3   Meiotic drive

Although previously contested due previous lack of empirical evidence (Coyne et al., 1991; Coyne and Orr, 1993), meiotic drive and its relation to hybrid sterility has slowly been gaining empirical support (McDermott and Noor, 2010). Meiotic drive is the process wherein an unequal segregation of genetic material during meiosis results in deviations from Mendelian ratios in gametes (Hurst and Pomiankowski, 1991; Presgraves, 2008). Drive elements are selfishly overrepresented in the population, even at the expense of the individual's fertility. As such, suppressor elements evolve alongside drivers, creating an arms race between the selfish element and its suppressor (McDermott

and Noor, 2010). It is more likely for a suppressor element to evolve on the autosome, instead of the small Y chromosome, to suppress the activity of the X-linked driver (Hurst and Pomiankowski, 1991; Atlan et al., 2003). How this system links to male hybrid sterility will involve an X-linked driver element and (most likely) an autosomal-linked suppressor. If a driver element is present on the X chromosomes and, during sperm development, reduces the presence or fertility of Y-bearing sperm, the over-representation of females over generations will lead to species extinction (McDermott and Noor, 2010). Between two separate subpopulations, the arms race between driver and suppressor is established over time. If, upon reintroduction of both diverging subpopulations, hybridization occurs, the established system breaks down. The suppressor of one population is unable to suppress the driver of the other population, leading to the production of unfit hybrids. Meiotic drive has been noted in a few species of *Drosophila* (Stalker, 1961; James and Jaenike, 1990; Jaenike, 1996), including *D. simulans* (Cazemajor et al., 1997). Cazemajor et al. (1997) further uncovered distorters in multiple X-linked loci and suppressor loci on all autosomes. Meiotic drive has also been noticed in sterile hybrids between fission yeast species, *Schizosaccharomyces pombe* and *S. kambucha* (Zanders et al., 2014). Here, three meiotic drive alleles, derived from *S. kambucha,* singly contribute to hybrid sterility via low spore viability (Zanders et al., 2014). This suggests that genetic conflicts due to meiotic drive may play a role in speciation in numerous systems.

## 1.3   Genetic basis of hybrid male sterility

### 1.3.1   Improper chromosome pairing

Pairing of the X-Y chromosomes in *Drosophila* depends on the presence of an rDNA intergenic spacer region that is embedded in the X heterochromatin and near the Y centromere (McKee *et* al., 1992). When deleted, this region, which comprises a 240-bp repeat, disrupts X-Y pairing and results in X-Y nondisjunction. Interestingly, the 240-bp repeat has been shown to differ among closely related species in the *D. melanogaster* subgroup (Lohe and Roberts, 1990). As such, differences in this 240-bp repeat between closely related species may provide a possible mechanism wherein interspecies hybrids possessing two different variants of the 240-bp repeat may result in mispairing of the X

and Y chromosome. This in turn results in improper segregation, thus giving rise to hybrid sterility and Haldane's rule.

### 1.3.1.1    Speciation gene case study - meiotic pairing and *Prdm9*

In mice, hybrids produced from interspecific mating between *Mus musculus musculus* and *M. m. domesticus* exhibit spermatogenic failure (Good et al., 2008). Further analysis found that improper pairing of homologous chromosomes occurred, causing arrested meiosis during prophase (Mihola et al., 2009). Mapping for this sterility resulted in the discovery of the *Prdm9* gene (Mihola et al., 2009), which encodes a DNA binding protein containing a zinc-finger array. This gene has histone methyltransferase activity (Hayashi et al., 2005). It targets recombination hot spots and thus targets the location of double stranded breaks (DSBs) during recombination (Baudat et al., 2010; Paravano et al., 2010). Further analysis found that re-programming the PRDM9 protein's binding site (at the Zn-finger array) in sterile hybrid males rescues their fertility (Davies et al., 2016). Thus, each allele of PRDM9 possesses its own preference for a recombination hotspot, which can affect DSBs, affecting the pairing of homologous chromosomes during meiosis. Asymmetrical PRDM9 binding will reduce fertility in hybrid males. It was postulated that meiotic drive may be the mechanism for differences in PRDM9 binding in subpopulations undergoing speciation (Davies et al., 2016). Since meiotic drive would favour mutations that permit disruption of PRDM9 binding, those mutations are passed along to the next generation and individuals with heterozygous PRDM9 binding affinities will have the non-mutant chromosome properly exhibiting DSBs and then repaired via copying of the chromosome with the mutation. This can result in the accumulation of mutations at the PRDM9 binding site and account for observable differences between subpopulations in PRDM9 binding (Davies et al., 2016).

### 1.3.1.2    Mismatched ploidy

Hybrid speciation is perhaps one obvious example of massive karyotypic differences leading to species establishment, with many examples in plants and animals (Mallet, 2007). Within the genus *Helianthus,* 3 hybrid species (*H. anomalus, H. deserticola, H. paradoxus),* arose from two parental species (*H. annuus, H. petiolaris*; Rieseberg, 1991),

wherein each hybrid exhibits differences in karyotype from their parents (Chandler et al., 1986). Genotypic mapping of these three hybrid species and their parents have revealed hybrid speciation arose due to massive changes in karyotypes (Rieseberg et al., 1995, Lai et al., 2005). Further analysis through QTL mapping has identified that these changes are responsible for hybrid sterility between hybrids and their parents (Lai et al., 2005).

In plant populations, wherein related diploid and tetraploid plants coexist, reproductive barriers such as hybrid sterility can evolve due to the "triploid block" (Thompson and Lumaret, 1992). For example, natural sympatric populations of diploid and tetraploid species of *Dactylis glomerata* have lead to the establishment of postzygotic isolation barriers due to the formation of sterile hybrid triploid offspring (Lumaret and Barrientos, 1990; Bretagnolle and Thompson, 1996). In response to the production of sterile triploids, some plants have adapted different flowering times, or have increased selfing (Levin, 1985; Van Dijk, 1991; Petit et al., 1997).

## 1.3.1.3    Chromosomal inversions

Changes in chromosomal arrangements may lead to a reduction in gene flow between populations and result in species formation, a mechanism that may allow for species isolation even when there is a lack of a physical barrier between populations (Levin, 2002). New chromosomal arrangements arising between populations contribute to the accumulation of genetic differences that, upon introduction within a hybrid, can lead to genetic incompatibilities and hybrid sterility (Rieseberg et al., 1999, Noor et al., 2001, Navarro and Barton, 2003).

Chromosomal inversions introduce a possible mechanism for speciation to occur in face of gene flow, especially for species whose ecological niches overlap. This mechanism has been extensively studied in sterile hybrid males produced between *Drosophila persimilis* and *Drosophila pseudoobscura*, where hybrid sterility mapped to chromosomal inversions (Noor et al., 2001). Further analysis found that high divergence was evident within and adjacent to these inversions between both species and allow recombination rate occurring at these regions (Stevison et al., 2011; McGaugh and Noor, 2012). Thus,

within these inverted regions, hybrid incompatibility factors may be harboured and will persist in each species' population, even with gene flow occurring in other regions of the genome (Noor et al., 2001; Rieseberg, 2001).

## 1.3.2    Gene translocation and duplication

Gene duplication and the degeneration of one paralog can result in hybrid sterility. When an ancestral population harbouring two duplicate genes (consider both fully functional, thus one is redundant) and is split into subpopulations, each population has the potential of losing one of the duplicated genes. The fates of each duplicate gene will likely differ between each subpopulation, as each population fixes alternative copies of the gene (Lynch and Force, 2000). If that gene is essential for each respective population's fitness, then genome hybridization after sufficient isolation from each other may result in sterility within a hybrid individual. This is further exacerbated if the alternative alleles are not functional as single copies or if the two alternative gene products interact negatively with each other (Werth and Windham, 1991). This proposed model, driven by any possible degenerative mutations and the silencing of one of the redundant genes, has been documented in plants and animals (Gottlieb and Ford, 1997; Force et al., 1999).

## 1.3.2.1    Speciation gene case study - transposition and *JYalpha*

Masly et al. (2006) demonstrated that divergence in gene function between species is not the only route for the evolution of hybrid sterility, and genes that jump within the genome – gene transposition – also have the potential to cause reproductive isolation. *JYalpha*, a gene that has been reported to undergo gene transposition, leads to hybrid sterility between *D. simulans*-*D. melanogaster* $F_2$-like hybrids (Masly et al., 2006). This gene is located on the fourth chromosome of *D. melanogaster* and on the third chromosome of *D. simulans*. Within a hybrid individual that possesses a homozygous *D. simulans* 4th chromosome and a homozygous *D. melanogaster* 3rd chromosome, and thus no copy of the *JYalpha* gene, mostly immotile sperm are produced, suggesting that this gene may have partial effect on a hybrid's fitness (Masly et al., 2006). It's important to note that in order to study the effects of *JYalpha* in sterile hybrid males, introgression lines were created. Thus *JYalpha* only affects $F_2$-like hybrids, suggesting possible recessive-

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recessive interactions, as parts of the hybrid's genome will be homozygous for one parental species. Although *JYalpha* does not provide a universal model for the evolution of hybrid sterility leading to reproductive isolation, this gene does demonstrate a previously overlooked mechanism for hybrid sterility (Masly et al., 2006).

## 1.3.2.2    Speciation gene case study - *odsH* and novel gene functions in hybrids

Perez et al. (1993) fine-mapped the X chromosome of *Drosophila mauritiana* to a region harbouring a gene, *Odysseus homeobox* (O*dsH;* also called *Ods-site homeobox*)*,* that contributes to hybrid male sterility between *D. simulans* and *D. mauritiana.* Further analysis of *OdsH* revealed the necessity of an additional and nearby gene in order to induce full sterility in a hybrid individual (Perez and Wu, 1995). The gene *OdsH* acts recessively in its contribution to hybrid sterility, and was shown to only induce sterility in *D. simulans*-*D. mauritiana* hybrids in a homozygous background, suggesting a possible recessive-recessive interaction is occurring (not recessive-dominant), thus this gene can only affect $F_2$ hybrids (not $F_1$ hybrids). Currently, an autosomal interactor with *OdsH* has yet to be identified.

*Odysseus* serves as an example of the importance of gene duplication in speciation. It has been shown the *OdsH* arose through a gene duplication event in *Drosophila* and at a high rate within the lineages that lead to the formation of the melanogaster group of species (Ting et al., 2004). Interestingly, the presence of *OdsH* within a hybrid background appears to affect production of sperm within males (Sun et al., 2004). When disrupted in a pure species background, *OdsH* has a minimal effect on the acceleration of sperm maturation, yet when functional alleles from divergent species are introduced within a hybrid, sperm production in males is drastically reduced, presenting a novel manifestation of *OdsH* function, rather than its gain or loss of function (Sun et al., 2004).

## 1.3.3    Sterility due to genetic divergence at the same locus

Sequence divergence between populations can establish reproductive barriers that keep species separate. Within the *sensu stricto* complex, *Saccharomyces cerevisiae* and its closest relative, *S. paradoxus,* exhibit no prezygotic isolation and are capable of

producing viable interspecific offspring. However, these F₁ hybrids produce few viable spores (Naumov, 1987). *Saccharomyces* species all have entirely co-linear genomes (Fisher et al., 2000) and thus changes in chromosomal arrangements (e.g. translocations, inversions, etc.) are not contributors to the production of these sterile hybrids. Furthermore, altering 2N hybrids into 4N allo-tetraploids induced higher levels of fertility, thus reducing the possibility that genetic incompatibilities are responsible for the production sterile hybrids (Greig et al., 2002).

The frequency and success of genetic recombination depends largely on the proper formation of the two homologous DNA duplexes. The mismatch repair system plays a role in the activity of genetic recombination, such that its inactivity can greatly reduce recombination, as seen in organisms with divergent genomes (Rayssiguier et al., 1989). As such, it was speculated and then tested whether sterility between these two species may be a by-product of sequence divergence, reducing recombination and impacting proper segregation during meiosis (Hunter et al., 1996). Hybrids with dysfunctional mismatch-repair systems exhibited higher levels of fertility and an increase in homologous recombination. Furthermore, introduction of chromosome 3 from *S. paradoxus* into the genetic background of *S. cerevisiae* resulted in a reduction of meiotic recombination, suggesting that sequence divergence can affect recombination via the mismatch repair system (Chambers et al., 1996).

## 1.3.3.1    Speciation gene case study - genetic divergence and *SaM/SaF*

Semi-sterility in hybrid males is seen within interspecific crosses between Asian rice subspecies, *Oryza sativa indica* and *O. sativa japonica.* Within the *Sa* locus, two adjacent genes, *SaM* and *SaF*, are expressed as different alleles for each subspecies. *O. sativa indica* cultivars have *SaM⁺SaF⁺* haplotype, while *O. sativa japonica* have *SaM⁻SaF⁻* haplotype (Long et al., 2008). Semi-sterility in males is achieved by pollen abortion when carrying the *SaM⁻* allele. The evolution of these two separate haplotypes may have arisen in a two-step introduction of nucleotide variation via genetic drift. The model for semi-sterility between these two subspecies is through a two-gene/three-component interaction (Long et al., 2008; Ouyang et al., 2010). Here, the absence of either *SaM⁺, SaM⁻, SaF⁺*

results in fertility, as deriving from genetic divergence. Sterility arises via selective transport of $SaM^+SaF^+$ proteins from their own microspores into the microspores carrying the protein from $SaM^-$(Long et al., 2008).

## 1.3.4    Centromeric divergence and segregation distortion

As previously described (see section 1.2.3), the genomic conflict between meiotic drivers and suppressors establishes a unique system that is compatible only within the population the system has evolved under (McDermott and Noor, 2010). If the driver and its suppressor are involved in meiotic drive, and if these elements are present on the sex chromosomes, it is to be expected that interspecific hybrids are more susceptible to defects that will result in sterility (Frank, 1991a; Hurst and Pomiankowski, 1991; Tao and Hartl, 1991).

A possible alternative explanation for meiotic mis-segregation in hybrids may be due to centromeric divergence. Centromeres, which are comprised of heterochromatin, recruit the machinery necessary for faithful segregation of meiotically-dividing chromosomes (Dernburg et al., 1996). Although this machinery is highly conserved across species, evidence has shown that heterochromatic centromeres are rapidly evolving, and proteins that associate to the centromere show signatures of strong adaptive pressure (Csink and Henikoff, 1998). This suggests a possible co-evolution of these genes alongside their associated heterochromatin (Malik and Henikoff, 2001; Brideau et al., 2006). The abundance and sequence of the repeats found within centromeres are also highly divergent between even closely related species (Lohe and Roberts, 1988), and thus may be one of the first regions of the genome to diverge in sequence after new species arise. Due to the greater dissimilarity between centromeric DNA of the X and Y chromosome, these chromosomes are more likely to be unable to segregate in interspecies hybrids (Henikoff et al., 2001). This segregation failure may be due to the inability of the associated proteins involved in the segregation machinery to recognize the target sequence. This provides another possible mechanism for hybrid sterility and Haldane's rule. The rapid evolution of non-coding DNA may therefore be responsible for the establishment of postzygotic reproductive barriers, rather than individual gene product incompatibilities between the X chromosome and autosomes.

## 1.3.4.1　Speciation gene case study - *Overdrive*

The gene *Overdrive* (*Ovd*), was shown to not only cause sterility, but also segregation distortion in $F_1$ hybrid males from *D. pseudoobscura bogotana* females mated to *D. pseudoobscura pseudoobscura* males, suggesting a possible linked genetic basis between the two processes (Phadnis and Orr, 2009). *Ovd* contains a MADF domain, which is involved in sequence specific DNA binding and potential serves as a transcription factor during *Drosophila* development (Phadnis and Orr, 2009; England et al., 1992). What is of interest in this species pair is that they are young species and have likely not yet accumulated a high quantity of genetic incompatibilities – a dilemma that researchers face in older species (Matute et al., 2010). It is known that *Ovd* has undergone rapid evolution (Phadnis and Orr, 2009). The genes that interact with *Ovd* to give rise to both hybrid sterility and segregation distortion have, however, yet to be determined. Further mapping has been employed to identify regions along the X chromosome and autosomes that may interact with *Ovd* to induce hybrid sterility and segregation distortion in this species pair (Phadnis, 2011).

## 1.3.5　Transposable elements

Transposable element (TE) movement, which can be triggered via hybridization, may result in genome restructuring, inevitably resulting in sterility (McClintock, 1984). Although many examples of TE movement and hybrid sterility can be found in plants, some animal species have shown intraspecific hybrid sterility (Michalak, 2009). Factors that may control, or repress, TEs are small RNAs found within the maternally inherited cytoplasm. Two examples of the effects of maternal cytoplasm in hybrid sterility can be found in *Drosophila* intraspecific sterile hybrids. Within intraspecific hybrids made among strains of *D. melanogaster,* fathers carrying TEs and mothers lacking these TEs produce sterile offspring. With the reciprocal cross, mothers carrying TEs and fathers without them, hybrids that are produced remain fertile (Yannopoulous and Stamatis, 1987). Stamatis, 1987). Further evidence of the effects of maternally transferred cytoplasm on hybrid fertility can been with the transfer of small RNAs and their role in repressing TEs within intraspecific crosses of *D. virilis*. Here, hybrid male sterility is facilitated with the retrotransposon element *Penelope*, which can induce high levels of

transposition of other TEs in the genome in strains that do not normally possess *Penelope* (Evgen'ev et al., 1997). Mothers produce sterile hybrid males if they are unable to provide the *Penelope*-derived siRNA (Blumenstiel and Hartl, 2005).

Mobile elements, upon mobilization, have been previously demonstrated to affect fertility in hybrid individuals (see above; Petrov et al., 1995). A possible mechanism for the suppression of these mobile elements within an individual would be methylation of the CpG sites found within promoters, arising due to the evolutionary arms race between the host and parasite (Bestor and Tycleo, 1996). As such, any given population along their evolutionary pathways could establish specific and unique methylation patterns for suppression of parasitic mobile elements. If two populations derived from an ancestral population are separated and their genomes are reintroduced after the formation of this system, hybrids may exhibit a reduction in DNA methylation, allowing for the mobilization of these elements. If the activity of these mobile elements affects fitness, specifically fertility, the hybrid individuals will experience a reduction in fitness. This theory has been examined within interspecific kangaroo hybrids: *Macropus eugenii* x *Wallabia bicolor.* These two parental species produce sterile hybrid males that do not produce sperm. Further analysis found that under-methylation of retroviral elements in hybrids lead to their amplification within the heterochromatic centromeres (Waugh et al., 1998), which may lead to sterility within these hybrids (the extent to heterochromatin and sterility remains unknown).

## 1.3.6   Nuclear-mitochondrial interactions

Hybrid breakdown may not be limited to nuclear-nuclear incompatibilities. Potential nuclear-mitochondrial interactions may also be sensitive to interspecific hybridization (Perrot-Minnot et al., 2004; Zeyl et al., 2005). Since mitochondrial inheritance is maternal and mitochondrial DNA (mtDNA) evolves rapidly, it's possible for hybridization to result in sterility due to an incompatible interaction between the mitochondrial and nuclear genomes of divergent populations. The proteins produced by the mitochondrial genome have an intricate interaction with nuclear gene products, resulting in the promotion of proper respiration and proper functioning of the electron transport chain. Co-adaptation between mito-nuclear genes within allopatric species

establishes a system that can be disrupted upon introduction of a haploid genome from another population, as seen in marine copepods (Rawson and Burton, 2002). Within each population, the nuclear gene, cytochrome *c,* interacts more efficiently with the respective mitochondrial gene, cytochrome *c* oxidase, of their respective population. Here, population differentiation is established from three amino acid substitutions in cytochrome *c,* suggesting a sensitive system that, upon hybridization, can affect the electron transport chain and lower hybrid fitness (Rawson and Burton, 2002).

### 1.3.6.1 Speciation gene case study - mito-nuclear incompatibility between *OLI1* and *AEP2*

Diploid hybrids produced from an interspecific mating between *Saccharomyces cerevisiae* and *S. bayanus* are capable of reproducing asexually, however, their spore viability is low (<0.5%), indicating the presence of postzygotic isolation. To uncover the genetic incompatibilities within an interspecific hybrid, Lee et al. (2008) screened chromosome replacement lines for genes that contribute to the sterility of these hybrids. They found that the *S. bayanus AEP2* gene does not properly translate *S. cerevisiae* mitochondrial *OLI1* mRNA (subunit of the ATP synthase complex; Finnegan et al., 1991), and thus *OLI1* is unable to properly function within the mitochondrial background of *S. cerevisiae* resulting in a respiratory defect (Lee et al., 2008). Furthermore, AEP2 may be interacting with the 5`-UTR region of the *OLI1* mRNA during translation (Ellis et al., 1999), providing a target region for divergence. Further analysis supported this, finding highly divergent sequences at the 5`-UTR of the *OLI1* mRNA between *S. cerevisiae* and *S. bayanus* (Lee et al., 2008; Chou and Leu, 2010).

### 1.3.7 Cytoplasmic incompatibility due to endosymbionts

Endosymbionts and their invasion within a population may serve as mechanism for the evolution of reproductive barriers and speciation. One of the most famous endosymbionts that has been studied for its role in speciation is *Wolbachia* (Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Giordano et al., 1995; Reed and Warren 1995; Bordenstein et al., 2001). Populations infected with *Wolbachia* typically exhibit a unidirectional effect on offspring produced from infected individuals mated to uninfected individuals (Yen and

Barr, 1971).  Here, uninfected females that are mated to infected males with *Wolbachia* will mostly produce progeny that die as embryos; the reciprocal mating event produces viable offspring. As such, cytoplasmic incompatibility derived from infected cytoplasm (in eggs) and uninfected cytoplasm (in sperm) creates a developmental defect in the offspring produced. Cytoplasmic incompatibility via *Wolbachia* infection appears to be evolutionarily conserved at the functional level, as strains of *Wolbachia* found in one genus are capable of inducing offspring inviability if injected into species of another genus (Braig et al., 1994).

Cytoplasmic incompatibility manifests during early stages of mitosis in embryogenesis, wherein the paternal genome improperly decondenses, degenerates, and the haploid individual that is left dies (Reed and Werren, 1995; Lassy and Karr, 1996; Callaini et al., 1997). A common example of how hybrid inviability is a by-product of *Wolbachia* infection is the interspecific crossing among three species of wasp, *Nasonia: N. vitripennis*, *N. giraulti*, and *N. lonicornis* (Bordenstein et al., 2001). All three species are infected with different strains of *Wolbachia* and exhibit cytoplasmic incompatibility when one species is mated with another species, except one of two outcomes may occur: diploid females are converted into haploid males (Reed and Warren 1995) or offspring die as embryos (Bordenstein et al., 2001).

An instance wherein cytoplasmic incompatibility occur bidirectionally involves multiple strains of *Wolbachia* infecting different populations. This has been observed in both *Nasonia* (Breeuwer and Werren, 1990), as well as *Drosophila* (O'Neill and Karr, 1990). In *Drosophila,* if a female is infected with one strain of *Wolbachia,* she will not be immune to another strain of *Wolbachia.* Therefore, an infected female mated to a male infected with a different strain of *Wolbachia* will produce offspring that die before the individual reaches sexual maturation, regardless of the direction of the cross between the two populations (Hoffman and Turelli, 1997). The cellular basis of cytoplasmic incompatibility has yet to be determined.

But how does *Wolbachia* infestation within a population lead to speciation? One of the strongest examples of the impact *Wolbachia* infection has on speciation is interspecific

hybrids produced from infected *Drosophila recens* mated to uninfected *D. subquinaria* (Shoemaker et al., 1999). Here, *D. recens* females mated to *D. subquinaria* males produces inviable hybrids, whereas the reciprocal cross produces viable hybrids. However, strong prezygotic barriers greatly reduce the likelihood of mating, and thus producing hybrids at all, when *D. subquinaria* females are mated to *D. recens* males. This suggests *Wolbachia* related cytoplasmic incompatibility may play a role in reinforcing the reduction in gene flow between *D. recens* and *D. subquinaria* (Shoemaker et al., 1999). It is important to note, however, that males produced from either direction of interspecies crossing between *D. recens* and *D. subquinaria* are sterile (Shoemaker et al., 1999). As such, cytoplasmic incompatibility may not be the sole mechanism for reproductive isolation between *D. recens* and *D. subquinaria.*

Finally, *Wolbachia* related cytoplasmic incompatibility causes lethality but does not appear to cause hybrid sterility (Giordano et al., 1995; Bordenstein et al., 2001). Furthermore, there have been no accounts of heterogametic female cytoplasmic instability, to date (Coyne and Orr, 1999; Bordenstein, 2003; Keeling et al., 2003). As such, endosymbionts, such as *Wolbachia,* may not play a singular role in postzygotic isolation, but may contribute to postzygotic isolation in combination with other mechanisms.

## 1.4     Cellular basis of hybrid male sterility

### 1.4.1     Spermatogenesis in *Drosophila*

As species within the genus *Drosophila* were under study for this dissertation, and hybrid sterility affects the heterogametic sex, my primary focus will be on the stages of male spermatogenesis that can be targets for hybrid sterility factors.

In *Drosophila,* spermatogenesis can be subdivided into three main stages: pre-meiosis, meiosis, and spermiogenesis (Figure 1.2). Previous studies of spermatogenesis defects in sterile hybrid males have suggested that most defects occur during spermiogenesis, wherein sperm cells do not properly separate into single, mature sperm and remain bundled and interconnected (Kulathinal and Singh, 1998; Civetta, 2016). These findings suggest that hybrid sterility factors may be affecting the individualization or motility of

sperm. Although much of the initial cytological analysis of sterile hybrids suggest errors in spermiogenesis (Kulathinal and Singh, 1998), it is important to note that further dissection of each individual stage of spermatogenesis was not performed. It is possible that errors may have arisen in early stages of spermatogenesis, but bypass stage-specific arrest until spermiogenesis. Thus, revisiting morphological and cellular analysis of sterile hybrid males, and examining each major stage of spermatogenesis, would be beneficial for refining our understanding of the stage at which spermatogenesis failure is initiated.
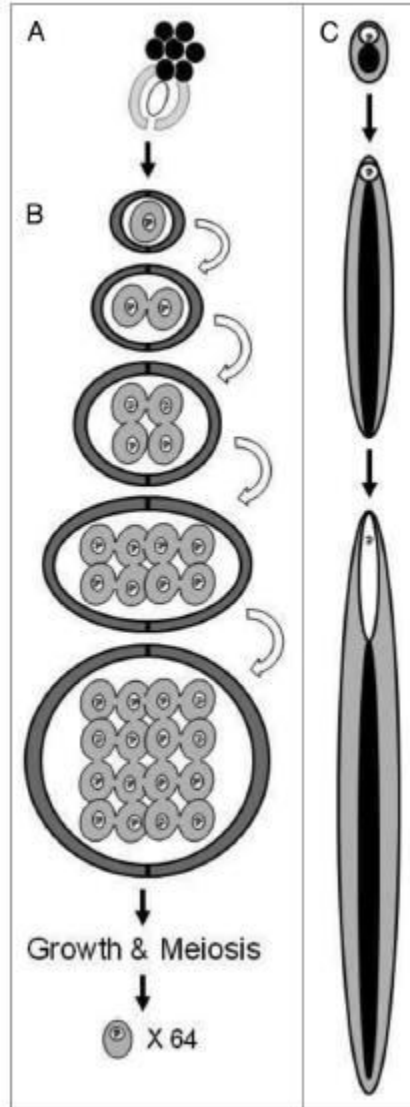
Figure 1.2. Spermatogenesis in *Drosophila melanogaster* (image taken from Kanippayoor et al., 2012). A) Somatic hub cells (black) associate with GSCs (white) and CPC (light gray). B) GSCs and CPCs produce a spermatogonium (gray) that is surrounded by a cyst cell (dark gray). The primary spermatogonium will undergo mitotic divisions to produce 16 primary spermatocyte cells that are interconnected by cytoplasmic bridges. These spermatocytes will then undergo meiosis, producing a cyst with 64 spermatids. C) The 64 mitochondria produced clump together to form the nebenkern (black). The sperm cell and the nebenkern elongate through to the stages of spermiogenesis.

## 1.4.1.1    Pre-meiosis

In *Drosophila,* the initial stage of spermatogenesis involves the differentiation of cells from a stem cell niche, to the eventual maturation of that cell into a primary spermatocyte. The apical end of the testes contains the germline proliferation center, consisting of the "hub" cells, germline stem cells (GSC), somatic cyst progenitor stem cells (CPC), and spermatogonia (Fuller, 1999). Here, the two population of stem cells are thought be under the control of the JAK-STAT pathway, permitting both the differentiation and maintenance of each stem cell niche (Fuller and Spradling, 2007). One possible target for this pathway is ZFH-1 (zinc-finger homeodomain-1), which is functionally described as a transcriptional repressor that maintains CPCs, although the specific function is poorly understood (Terry et al., 2006). If hybrid sterility factors were to affect this stage of spermatogenesis, it would be expected that cells from the germline proliferation center would show improper maintenance and differentiation of the two stem cell populations.

It's important to note that although the process of meiosis is conserved across all species in the genus *Drosophila*, the number of pre-meiotic mitotic divisions is species specific. For example, species of the *D. melanogaster* subgroup undergo four mitotic divisions to produce 16 primary spermatocytes, whereas species within the *D. pseudoobscura* group undergo five mitotic divisions to produce 32 primary spermatocytes (Pantazidis et al., 1992; Scharer et al., 2008).

## 1.4.1.2    Meiosis

Meiosis begins with the diploid primary spermatocytes that eventually end as haploid cells. In *Drosophila,* since mitotic divisions differ among species, the number of haploid sperm produced after meiosis also differs. For example, species of the *D. melanogaster* subgroup and *D. pseudoobscura* group produce 64 sperm and 128 sperm, respectively (Pantazidis et al., 1992; Scharer et al., 2008). Furthermore*,* primary spermatocytes undergo meiosis with incomplete cytokinesis, such that sperm cells remain interconnected by cytoplasmic bridges (Fuller, 1993). Here, transcription is high, as many proteins necessary for spermiogenesis after meiosis is complete are transcribed within the

primary spermatocyte and are stored translationally repressed until after meiosis (reviewed in: White-Cooper, 2010). Some examples of genes transcribed in primary spermatocytes with known protein function include genes involved in mitochondrial fusion (*fzo;* Hwa et al., 2002), meiotic spindle and axoneme (cytoskeletal structure of the sperm tail) formation (*βtub85D*; Kemphues et al., 1979), faithful execution of meiosis (*twine;* White-Cooper, 1996), chromatin structure (*Dpy-30L2*; Vardanyan et al., 2008), and transcription/translational regulation (*aly, boule, topi*; White-Cooper et al., 2000; Eberhart et al., 1996; Perezgazga et al., 2004).

An overall trend of misexpressed of genes has been noted in interspecific hybrids in *Drosophila* (Michalak and Noor, 2003; Haerty and Singh, 2006; Moehring et al., 2007). Genes in sterile male hybrids express lower levels of transcripts, with the exception of genes on the X chromosome that saw overexpression (Moehring et al., 2007). Of the genes described above that are involved in spermatogenic meiosis, *aly, topi,* and *βtub85D* are all underexpressed in adult sterile males (Moehring et al., 2007). However, it should be noted that misexpression of genes in hybrids has recently been contested as an underlying mechanism of sterility, as fertile male hybrids also have high levels of spermatogenesis-related gene misexpression (Gomes and Civetta, 2014).

## 1.4.1.3    Spermiogenesis

One of the most notable changes during sperm development is the transition from round spermatid to an elongated sperm cell during the process of spermiogenesis, which occurs after meiosis is complete. Here, the sperm tail forms and elongates, DNA is repackaged and further condensed within the sperm head and sperm individualization occurs. Further analysis of misexpressed genes in sterile hybrid males have targeted genes involved in spermiogenesis (post-meiotic), with some genes misexpressed across different interspecies pairs (Michalak and Noor, 2004; Noor, 2005; Moehring et al., 2007; Sundararajan and Civetta, 2011; Ferguson et al., 2013).  To date, none of the misexpressed genes identified in these studies have been directly tested for their effects on sterile hybrid males. However, one gene that is transcribed during spermiogenesis (*soti*) has been noted to directly affect fertility within a species. Homozygous mutants for *soti*, are sterile with no sperm individualization (Barreau et al., 2008).

### 1.4.2 Future directions for the study of male hybrid sterility in *Drosophila*

Studies attempting to dissect the genetic basis of hybrid sterility and identify a general underlying mechanism have had conflicting results, as a lack of evidence exists for both the Dominance theory and meiotic drive (McDermott and Noor, 2010; Presgraves, 2010). Furthermore, less attention has been given in understanding, at the cellular level, where errors may arise in sterile hybrid males in *Drosophila* (but see Kulathinal and Singh, 1998; Hardy et al., 2011). Perhaps the key to uncovering incompatible loci for male hybrid sterility is to first identify at which stage errors arise during spermatogenesis. Secondly, a genus-wide comparison for spermatogenic failures in sterile male hybrids among different interspecies hybrids in *Drosophila* would highlight general patterns. If a phenotype is noted across all (or many) interspecies sterile hybrids, genetic mapping for this phenotype could be performed to identify the loci involved.

## 1.5 Overview of dissertation

In this dissertation, I explore errors occurring during spermatogenesis and identify genomic regions contributing to sterility in interspecific hybrid males in *Drosophila.* My primary research goals in undertaking this project are: 1) identify errors in spermatogenesis across multiple interspecies pairs by examining testes and sperm morphology of sterile hybrid males to identify whether failure occurs at the same stage of spermatogenesis, 2) determine if sperm are dead or alive with low mitochondrial activity of sperm-producing sterile hybrid males, 3) assess if protamine allelic expression is the same in sterile male hybrids and in males from pure species, 4) locate any genetic factors that would give rise to a previously-uncharacterized phenotype ("needle-eye") of sterile hybrid male sperm.

In Chapter 2, I examine testes cellular morphology of sterile hybrid males in *Drosophila.* Here, I examined if spermatogenesis fails at similar stages across multiple interspecies sterile hybrid males. Using various microscopy techniques, I examine testes morphology, chromosome segregation after meiosis I, number of sperm produced after spermatogenesis, ultrastructural analysis of sperm, and the effects of aging on sperm head

length in sterile male hybrids from three interspecific crosses in *Drosophila: D. simulans/D. mauritiana*, *D. pseudoobscura/D. persimilis*, and *D. arizonae/D. mojavensis*. I find that hybrid males have smaller testes than parental pure species males, with sperm bundles that are shorter and more disorganized. After meiosis I, chromosomes do not separate between daughter cells in hybrids, suggesting non-disjunction has occurred. The occurrence of non-disjunction is supported by additional lines of evidence. The number of sperm produced after spermatogenesis is roughly half that of parental pure species. Each sperm within the bundle in hybrids from interspecific crosses between *D. simulans/D. mauritiana* and *D. pseudoobscura/D. persimilis* possess two sperm tails and two undifferentiated mitochondria. Finally, sperm head length is aberrant in sterile hybrid males aged for 6 days, suggesting improper nuclear packaging. This study further refines the observations of Kulathinal and Singh (1998), suggesting that errors may be occurring during meiosis, causing the downstream affects during spermiogenesis that they observed.

In Chapter 3, I examine sperm viability and sperm mitochondrial activity (a factor potentially underlying lack of motility) in sterile hybrid males from the same three interspecific crosses examined in Chapter 2. As most hybrids produced from these interspecific crosses produce non-motile sperm (with the exception of one direction of one cross, where no sperm are produced; Kulathinal and Singh 1998), hybrid sperm may be dead or have undergone apoptosis. In addition, sperm from sterile hybrid males have undifferentiated mitochondria (see Chapter 2); therefore, the lack of motility in sterile hybrid males may be due to non-functioning mitochondria. Using an assay that detects intact and functional cell membranes, an indicator of cell viability, all sperm are observably viable in sterile hybrids and in parental pure species. Furthermore, using an indicator of functional mitochondria, mitochondria along sperm tails were functional in both sterile hybrid males and parental pure species. This suggests that sterility is not a by-product of poor sperm membrane structure nor non-functional mitochondria, warranting further examination of the sperm head.

In Chapter 4, I examine allelic expression of protamines in sterile hybrid males and pure species, with a focus on only one interspecies cross: *D. simulans* mated to *D. mauritiana*.

Within species comparisons (i.e., *D. simulans* mated to *D. simulans* and *D. mauritiana* mated to *D. mauritiana)* of protamine allelic expression uncovered a bi-allelic expression. In other words, the alleles from both homologs contribute to the expression of protamines used in the packaging of DNA in the sperm head. Sterile hybrid males produced from *D. simulans* and *D. mauritiana* also exhibit bi-allelic expression, thus any abnormalities in sperm head packaging are not due to one allele expression of protamines in hybrids. However, it could potentially still be due to a negative interaction between the protamines produced by the two species' alleles within a single hybrid individual.

In Chapter 5, I identify a novel sperm phenotype found in sterile hybrid males of all three interspecies crosses studied. This new phenotype was called "needle-eye" sperm. I also uncovered the genetic regions responsible for the formation of needle-eye sperm in sterile hybrid males produced from *D. simulans* and *D. mauritiana.* This was done through phenotype-based introgression and recombination backcrossing for 10 generations to either *D. simulans* or *D. mauritiana.* A bulk segregation analysis was performed by pooling males for each backcross into one of two samples: males that produce needle-eye sperm, males that do not produce needle-eye sperm. Samples were sequenced using Illumina technology and the raw sequences were analyzed using the *p*henotype-based *s*election and *i*ntrogression followed by whole-genome re*seq*uencing (PSIseq) pipeline. Genetic regions that are linked to the formation of needle-eye sperm contain a handful of genes in males produced from backcrossing to *D. mauritiana,* but further refinement of introgressions is needed for males produced from backcrossing to *D. simulans* in order to identify candidate genes.

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# Chapter 2

## 2    Cellular abnormalities and spermatogenetic errors in sterile hybrid males in *Drosophila*

The stages of spermatogenesis have been thoroughly examined within multiple species of *Drosophila*. Interspecific matings between two species of *Drosophila* produce hybrids with non-motile sperm. Characterizing the cellular basis of hybrid male sterility across multiple *Drosophila* species can potentially identify mechanisms for the evolution of hybrid sterility in this genus. The purpose of this study is to identify the stage at which spermatogenesis fails in sterile hybrid males, and do so for three different groups within the genus *Drosophila*. Here, I identified failures occur during meiosis I in all three groups, wherein chromosomes appear incapable of proper segregation, leading to half the number of sperm produced during spermatogenesis in all three interspecies crosses. Specifically, individual sperm cells derived from hybrid males carry two tails and non-differentiated mitochondria. In addition, sterile hybrid males aged for six days exhibit rapid nuclear de-condensation in all three crosses. Lastly, since meiotic failure was noted in all three interspecies pairs studied, failure during meiosis (and the downstream effects of this failure) may potentially be a general phenomenon in *Drosophila*.

## 2.1   Introduction

### 2.1.1   Spermatogenesis and speciation

Hybrid sterility is an intrinsic postzygotic isolating barrier that reduces gene flow between species, keeping them separate and distinct groups. According the Bateson-Dobzhansky-Muller incompatibility model, herein referred to as the BDM model, genetic differences accumulate due to mutation and the evolutionary forces that act on them (Bateson, 1909; Dobzhansky, 1934; Muller, 1940; Lynch and Force, 2000). Within each population, changes over time to the genetic make-up that negatively interact with other loci are selected against. Compatible interacting loci within a population may, however, be incompatible with loci of a foreign genome. If two populations that follow along separate evolutionary paths are later re-introduced, incompatible loci will have the first

opportunity to interact within the F₁interspecies hybrid. Genetic incompatibility within a hybrid individual may affect their reproductive systems, rendering the hybrid sterile, thus reducing the hybrid's fitness (Bateson, 1909; Dobzhansky, 1936; Muller, 1940) and serve as a mechanism for reproductive isolation.

Among many interspecific crosses, a notable trend (herein called Haldane's rule, HR) has been observed, wherein if only one sex is sterile, it's almost always the heterogametic hybrids that are sterile, as opposed to the homogametic hybrids (Haldane, 1922; Coyne and Orr, 1989; Coyne and Orr, 1997; Presgraves and Orr, 1998; Schilthuizen, 2011). The widespread observation of HR raises the possibility that there may be a common underlying basis of hybrid sterility. Extensive research has been put forth to understand how the underlying genetic basis of hybrid sterility relates to Haldane's rule (Presgraves, 2010; Maheshwari and Barbash, 2011; Delph and Demuth, 2016).

### 2.1.2    Theories on the genetic relationship between hybrid sterility and Haldane's rule

The most widely accepted model for the genetic basis of hybrid sterility is the Dominance theory, which suggests that random divergent mutations arising in the lineages of both species are incompatible when they come together within a hybrid individual (Bateson, 1909; Dobzhansky, 1934; Muller, 1940; Orr and Turelli, 1995). In its simplest form, the incompatible interaction between a recessive X-linked locus derived from one parental species and a dominant autosomal-linked locus derived from another parental species affects the reproductive system of the hybrid, rendering them sterile (Orr and Turelli, 1995). While a single pair of interacting loci can theoretically cause sterility, these genetic incompatibilities increase and accumulate over time, which introduces difficulties in identifying the initial interacting loci responsible for HR (Orr, 1995; Matute et al., 2010). Although genetic mapping studies have identified individual genes that contribute to interspecies hybrid sterility, particularly within the genus *Drosophila* (Perez et al., 1993; Perez and Wu, 1995; Masly et al., 2006; Phadnis and Orr, 2009; Phadnis, 2011), few lend support to this model (Presgraves, 2010).

The second most favoured model, meiotic drive, was initially criticized and discounted as a favourable explanation for hybrid sterility and HR (Coyne and Orr, 1993; Charlesworth et al., 1993; Coyne and Orr, 2004). However, an increase in empirical data suggests that meiotic drive may in fact play an important role in HR and the evolution of hybrid sterility (McDermott and Noor, 2010). A selfish driver located on the X chromosome can be introduced and amplified within a population, so long as a suppressor for the driver arises in time to override the deleterious effect the driver has on an individual, such as through reduced fertility (Edward, 1961). Selfish drivers that invade a population, disrupt sex ratios, and/or deleteriously affect an individual promotes selection for the evolution of a suppressor for the driver (Crow, 1991). However, additional drivers linked to meiotic drive may arise within the population that are capable of evading old and established suppressors (Hall, 2004; Wilkinson et al., 2014), necessitating the evolution of yet another suppressor. As such, a rapid, intergenomic conflict is establish between drivers and suppressors within a population, increasing the likelihood of driver-suppressor mis-match in hybrids formed between divergent populations. Meiotic drive and suppressor genes are more likely to evolve on sex chromosomes (Hurst and Pomiankowski, 1991), and as such if an X-linked driver responsible for meiotic drive has a Y-linked suppressor, disruption in sex ratios and sterility would be observed in interspecific hybrids that received a Y chromosome from another species that did not evolve that particular suppressor (Frank, 1991; Hurst and Pomiankowski, 1991).

### 2.1.3   Stages of spermatogenesis in *Drosophila*

Exploring hybrid sterility from a cellular approach, rather than a genetic mapping approach, may yield new insights into the underlying basis of interspecies sterility. The cellular process of spermatogenesis has been studied in a variety of species (Clouthier, 1996; L' Hernault, 1997; Fuller, 1998; Birkhead et al., 2008), including within the model system of *Drosophila*. The production of male gametes has been well characterized and studied across many *Drosophila* species (e.g., Fuller, 1998; Scharer et al., 2008; Davis and Fuller, 2009). Furthermore, the major stages of cell differentiation and transformation that occur during the process of spermatogenesis are easily identifiable.

Spermatogenesis is highly conserved across the *Drosophila* genus and can be subdivided into three main stages: pre-meiosis, meiosis, and post-meiosis. During the pre-meiotic stage, a central hub located at the apical end of the testes undergoes an asymmetrical division, producing one stem cell that will return to the hub and one gonialblast that will continue through the process (Davis and Fuller, 2009). A species-specific number of mitotic divisions (five to eight) produce spermatogonia that later develop into primary spermatocytes (Pantazidis et al., 1992; Scharer et al., 2008). The mitotic events do not undergo complete cytokinesis, thus creating a series of interconnected spermatocytes within a single syncytium (Davis and Fuller, 2009). Primary spermatocytes then enter into meiosis, wherein the diploid genome splits to a haploid genome (Davis and Fuller, 2009).

The events that occur during the post-meiotic stage of spermatogenesis in *Drosophila* involve the drastic transformation of a round haploid cell into a species specific haploid sperm shape that is fully motile. This transformation process also includes repackaging the DNA from histones onto protamines (Kanippayoor et al., 2013), condensing the DNA to a level of compaction that properly packages into the small volume of the sperm head, a necessary property for a hydrodynamic sperm (Tokuyasu, 1972). Within the sperm bundle, mitochondrial fusion results in the formation and intertwining of the minor and major mitochondrial derivatives, or the Nebenkern (Tokuyasu, 1974; Hales and Fuller, 1997). Both mitochondrial derivatives move alongside the growing sperm tail. Due to the differences in mitotic divisions between *Drosophila* species, the number of sperm produced from one round of spermatogenesis differs. Specifically, *Drosophila arizonae, Drosophila mojavensis,* and species within the *Drosophila simulans* complex (e.g. *Drosophila simulans* and *Drosophila mauritiana*) undergo four mitotic divisions to produce 16 primary spermatocytes, giving rise to 64 sperm per sperm bundle (Zouros, 1991). In contrast, *Drosophila pseudoobscura* and *Drosophila persimilis* undergo five mitotic divisions to produce 32 primary spermatocytes, leading to 128 sperm per sperm bundle (Pantazidis et al., 1992; Scharer et al., 2008).

Abnormalities and errors arising during spermatogenesis have been noted and characterized at the cellular level, especially in *Drosophila melanogaster* (reviewed in:

White-Cooper, 2004). While one study has also examined cytological abnormalities within interspecies hybrids of *Drosophila* (Kulathinal and Singh, 1998), this study occurred prior to significant advances in imaging technology. Furthermore, the study performed by Kulathinal and Singh (1998) could not determine the exact stage at which spermatogenic failure occurred, and all of the species examined were from within a closely-related species complex (the *simulans* complex).

An examination of hybrid spermatogenic failure across diverse species pairs of *Drosophila* will allow for an assessment of where spermatogenic failure occurs across this well-studied genus. If the genetic basis of spermatogenic failure can occur at any stage, by any loci, as predicted by BDM (Bateson, 1909; Dobzhansky, 1936; Muller, 1940), then the stage at which failure occurs should be different among interspecies pairs. In other words, if mutation is randomly introduced into a population, and increase in allelic frequency of that mutation depends on evolutionary forces such as drift or natural selection that should vary between isolated populations, then most newly-arisen fixed allelic variants would be expected to be different in these isolated populations. In addition, how genetic incompatibilities manifest to affect hybrid fitness should be unique among different hybridization events of different species pairs (Bateson, 1909; Dobzhansky, 1936; Muller, 1940). If, however, the basis is not due to random genetic changes, but rather due to genetic changes in a very specific locus or subset of loci, or due to a specific stage of spermatogenesis being highly susceptible to cellular failure, then a trend may emerge. Such a trend would propose a potential generalized mechanism for postzygotic isolation, an observation not made since the introduction of Haldane's rule (Haldane, 1922).

## 2.1.4    Overview of study

This study aims to identify failures occurring during spermatogenesis across multiple interspecies pairs in the genus *Drosophila.* To obtain a broad view, I chose species spanning the *Drosophila* genus (Figure 2.1H). Since species accumulate divergent mutations over time (e.g., Orr, 1995; Matute et al., 2010), it is not surprising that more divergent species pairs usually produce hybrids with more severe sterility phenotypes, such as heavily atrophied testes and the absence of sperm, such as among *D.*

*melanogaster* and its sibling species in the *simulans* complex (Engles and Preston, 1979). The first criterion was that all sterile interspecies hybrids I examined still produce sperm, allowing for an assessment of the entire spermatogenic pathway. In the *D. simulans* complex, the sibling species *D. simulans* and *D. mauritiana* diverged approximately 260,000 years ago (Kliman et al., 2000) and are well studied in the field of hybrid sterility (Presgraves, 2010). The recently diverged (500,000 ya; Hey and Nielsen, 2004) species pair of *D. pseudoobscura-D. persimilis* can additionally be used as a model of whether hybrid sterility has a differential impact on sperm morphs as these species produce both fertilizing sperm (eusperm) and non-fertilizing sperm (parasperm) morphs in their ejaculate (Snook et al., 1994). Lastly, the species pair *D. arizonae* and *D. mojavensis* were chosen due to their distant phylogenetic relationship to the other two species pairs and their recent divergence time from each other (0.6-1.2 my; Ruiz et al., 1990; Reed et al., 2008). Furthermore, these three chosen interspecies crosses have been previously demonstrated to exhibit hybrid male sterility (Coyne and Orr, 1989). In this study, I examined and identified the stage at which spermatogenesis fails across three interspecies hybrids in *Drosophila.*

## 2.2 Materials and methods

### 2.2.1 *Drosophila* stocks and maintenance

The *Drosophila mauritiana* stock was collected by Christopher Austin from Mauritius in May, 2012. Transgenic flies that possess protamines fused to a green fluorescent protein (see below) were created and acquired from Dr S. Pitnick and Dr J. Belote (Manier et al., 2010). All other pure species *Drosophila* stocks were acquired from the *Drosophila* Species Stock Center (San Diego, CA): *Drosophila simulans* (FC; stock #14021-0251.165), *Drosophila pseudoobscura* (#114011-0121.149)*, Drosophila persimilis* (#14011-0111.49)*, Drosophila arizonae* (#15081-1271.00)*, Drosophila mojavensis* (#1501-1352.22). To setup interspecific mating, 5 female *D. simulans* were mated to 5 male *D. mauritiana.* The reciprocal cross was not performed, as male hybrids produced no sperm (Kulathinal and Singh, 1998) and the intent of this study is to examine sperm of sterile hybrid males. Both directions of interspecific crosses were performed to produce

sterile hybrid males between *D. pseudoobscura* and *D. persimilis.* Five of each species was used for mating. Only one direction of interspecific mating was performed between *D. mojavensis* and *D. arizonae* to produce sterile hybrid males. Although interspecies hybrids can be made within a laboratory setting, the number of mating events is much less than the other interspecies crosses examined when a single male is presented to a single female. This may be a by-product of behavioural isolation (Massie, 2006). As such, 5 *D. arizonae* females were mated to 15 *D. mojavensis* males. The reciprocal cross could not be performed as *D. mojavensis* females rarely mated with *D. arizonae* males and few sterile hybrid males were produced. All flies within the *simulans* and *mojavensis* complexes were maintained on standard Bloomington recipe cornmeal media (Bloomington Drosophila Stock Center); flies from the *pseudoobscura* group were maintained on Bloomington recipe banana media. All flies were housed at 24°C on a 14:10 light:dark cycle at 75% humidity.

## 2.2.2 Cytological assessment of pure species and interspecies hybrids in *Drosophila*

Testes of *Drosophila* species and interspecies hybrids that were no more than 2 days old were extracted in Testes Buffer (183m$M$KCl, 47m$M$NaCl, 10m$M$ Tris-HCl) and were lightly squashed under a cover slip. Overall gross testes morphology was observed using a light microscope and images were taken with a Nikon camera and analyzed with NIS-elements. To observe individual stages of spermatogenesis, testes of pure species or hybrid males were extracted in 50 µl PBS buffer solution from 1-4 day old flies. Testes were then transferred to 20 µl 45% acetic acid for 7 minutes. A cover slip was pressed on top of the sample, and excess acetic acid removed. Samples were visualized using phase contrast microscopy on a Nikon Eclipse E100 compound microscope.

## 2.2.3 Determining number and head size of sperm produced after spermatogenesis

The number of sperm produced after spermatogenesis was determined by counting the number of sperm within a single sperm bundle. Sperm bundle counts were determined for

*D. mauritiana, D. arizonae,* and *D. pseudoobscura,* as well as interspecific hybrids produced with *D. simulans, D. mojavensis* and *D. persimilis,* respectively. Transgenic *D. mauritiana* (*w; P{w8, ProtB-EGFP, w⁺}8A*), *D. simulans* (*w⁺; pBac{3xP3-EGFP, ProtB-EGFP}11B*), and *D. pseudoobscura* (*w⁻; pBac{3xP3-GFP, ProtB-GFP}27*), which expresses a protamine GFP fusion protein, were used in this experiment to count the number of mature sperm per sperm bundle. For *D. mojavensis, D. arizonae*, and their interspecific hybrids, sperm per sperm bundle was determined using 0.5µl/ml of DAPI (4',6-diamidino-2-phenylindole), a nuclear stain. Images of fluorescent sperm were taken using a Leica DMI6000 B inverted fluorescent microscope and further analyzed using MetaMorph Imaging software. A Welch's two-sample t-test was performed using R, as it is a more robust statistical analysis that can perform better than Student's t-test when sample sizes and variances are unequal. In addition, effect size was calculated using Glass' delta equation (Glass et al., 1981), as effect size quantifies the difference in size between the two groups.

To measure the sperm head length in *D. mojavensis, D. arizonae, D. pseudoobscura, D. persimilis,* and their interspecific hybrids, testes were stained with 0.5µl/ml of DAPI. Transgenic *D. simulans* and *D. mauritiana* flies that were used to determine sperm per sperm bundle were also used to determine sperm head length in each species and their interspecific hybrids. Images were taken using a Nikon Eclipse Ci-L upright fluorescent microscopy, equipped with a DS-Fi2 colour camera. Image acquisition software used was Nikon Elements D and sperm head length was measured using ImageJ. A Student's two-sample *t*-test or a Welch's two-sample *t*-test was performed using R and effect size was calculated using Glass' delta equation (Glass et al., 1981). As nulcei appeared similar in length in pure species and their hybrids (Supplementary Figure 1), only 5-10 males were scored for sperm head length.

## 2.2.4   Transmission election microscopy (TEM)

Transmission electron microscopy (TEM) was used to examine testes from parental species of *D. simulans* and *D. persimilis* and interspecific hybrids of *D. simulans* and *D. mauritiana* (*sim/mau*) and *D. pseudoobscura* and *D. persimilis* (*pse/per*). The testes of

hybrids between *D. arizonae* and *D. mojavensis* were previously imaged with TEM (Hardy et al., 2011).Testes were dissected in phosphate buffer, immediately transferred to 2.5% glutaraldehyde/3.0% paraformaldehyde on a siliconized cover, and incubated in the fridge overnight. Testes were postfixed in 2% $OsO_4$ and dehydrated in acetone and embedded in Epon resin. Testes in Epon resin were sliced into 60nm slices, mounted on a 400 mesh nickel grid and stained with 2% uranyl acetate and 2% lead citrate. Samples were imaged on a Philips CM10 TEM.

## 2.3    Results

### 2.3.1    Morphological observations of *D. simulans*, *D. mauritiana*, and their interspecific hybrids (*sim/mau*)

Subtle observable differences in gross testes morphology are seen in interspecific hybrids, *sim/mau* (Figure 2.1). Visually, *sim/mau* have short and disorganized sperm bundles compared to pure species (Figure 2.1A, 2.1B, 2.1C). No obvious spermatogenic errors were present in the testes of any hybrid in the stages preceding the production of primary spermatocytes (data not shown). In primary spermatocytes undergoing the first meiotic division, it was apparent that chromosomes were not equally separating between the two dividing cells, resulting in what appears to be non-disjunction, however, which chromosomes that were not properly separating could not be determined (Figure 2.1D, 2.1F). For interspecies crosses between *D. simulans* and *D. mauritiana,* 17 out of the 23 primary spermatocytes observed exhibited a non-disjunction event. Pure species, *D. simulans* (n=8) and *D. mauritiana* (n=10), did not exhibited a non-disjunction event. Fluorescent *in-situ* hybridization (FISH) was unable to be successfully performed in order to determine which chromosomes were lagging, and the creation of a *D. simulans* specific probe for each chromosome was unsuccessful.

The number of sperm produced after spermatogenesis in *sim/mau* (n=10) was roughly half the number of sperm produced in *D. mauritiana* (n=10; Figure 2.4). This decrease in sperm number difference is significant, with a large effect size (P<0.001, $\Delta$=10.62). Examination of *sim/mau* sperm tail ultrastructure reveals that individual sperm cells possess two sperm tails and each nucleus varies in size (Figure 2.1G). In addition, each

mitochondrial derivative has not differentiated into the major and minor forms (Figure 2.1G).

Aged (6 days old) *sim/mau* males (n=10) produce sperm with nuclei that are longer in length than that of young (1 day old) *sim/mau* hybrids (n=10; Figure 2.5). This difference is significant, with a large effect size (P<0.001, $\Delta$=1.784), suggesting sperm nuclear de-condensation occurs in hybrids aged for a short period of time. When comparing sperm head length of *sim/mau* (both young and aged) to parental males (both young and old, n=5 for both parental species), *sim/mau* produce sperm that are longer (Figure 2.6). The increased difference observed is significant and with a large effect size when comparing young *D. simulans* to young *sim/mau* (P<0.001, $\Delta$=2.96) and young *D. mauritiana* to young *sim/mau* (P<0.001, $\Delta$=3.07). The increased difference is also significant and with a large effect size when comparing aged *D. simulans* to aged *sim/mau* (P<0.001, $\Delta$=3.96) and aged *D. mauritiana* to aged *sim/mau* (P<0.001, $\Delta$=3.95).

## 2.3.2 Morphological observations of *D. mojavensis*, *D. arizonae* and their interspecific hybrids (*ari/moj*)

Observable redundant differences in gross testes morphology are seen in hybrid males, *ari/moj,* which are produced from *D. mojavensis* mated to *D. arizonae* (Figure 2.2A, 2.2B, 2.2C). All stages of spermatogenesis could be seen in *ari/moj* testes, however, sperm bundles visually appeared disorganized and shorter than that of the parental species (Figure 2.2C). Upon closer inspection of primary spermatocytes in *ari/moj* males*,* a similar phenomenon was seen in *sim/mau* hybrids (Figure 2.1E). In interspecies hybrids between *D. arizonae* and *D. mojavensis*, all primary spermatocytes observed (n=6) exhibited a non-disjunction event and one instance of a chromosomal bridge between two separating chromosomes (Figure 2.2E). A non-disjunction event was not observed in any primary spermatocytes undergoing meiosis in *D. arizonae* (n=5).

The number of sperm produced after spermatogenesis was also quantified for *ari/moj* hybrids (n=10; Figure 2.4). Similar to the observation noted for *sim/mau* hybrids, the number of sperm within a sperm bundle is roughly half the number than that of the

parental species, *D. arizonae* (n=10), and the difference was significant (P<0.001) with a large effect size (Δ=6.48).

Aged (6 days old) *ari/moj* hybrids (n=10) also exhibit longer sperm nuclei length than that of young (1 day old) *ari/moj* hybrids (n=10; Figure 2.5). This difference is significant (P<0.001), with a large effect size (Δ=2.63), suggesting rapid sperm nuclear de-condensation in *ari/moj* hybrids. Sperm head length of young *ari/moj* (n=10), have shorter nuclei than young parental males, *D. arizonae* (n=5) and *D. mojavensis* (n=5; Figure 2.6). The difference between young *ari/moj* and young *D. arizonae* is significant (P<0.05) with a large effect size (Δ=4.56). The difference between young *ari/moj* and young *D. mojavensis* is significant (P<0.05) with a large effect size (Δ=4.58). The difference between aged *ari/moj* and aged *D. Arizonae* is both significant (P<0.05) and with a large effect size (Δ=1.03), an observation also seen between aged *ari/moj* and aged *D. mojavensis* (P<0.05, Δ=1.07).

### 2.3.3 Morphological observations of *D. pseudoobscura*, *D. persimilis*, and their interspecific hybrids (*pse/per*)

The most striking difference in gross testes morphology compared to the parental species could be seen in hybrid males, *pse/per,* that are produced from *D. pseudoobscura* and *D. persimilis* (Figure 2.3C). Hybrid testes were visibly smaller in size prior to mounting and imaging with a cover slip as qualitatively observed upon imaging when compared to parental species (Figure 2.3A, 2.3B, 2.3C). Sperm bundles of *pse/per* are visibly more disorganized than those of the parental species (Figure 2.3A, 2.3B, 2.3C). The various stages of spermatogenesis are not clearly seen in *pse/per* testes, wherein sperm bundles appear the most unrecognizable of the three hybrid groups examined. Primary spermatocytes of *pse/per* testes (n=7) that have undergone the first meiotic division also exhibit non-disjunction, with one instance of chromosomes appearing joined and unable to separate (Figure 2.3F). No non-disjunction events were observed in primary spermatocytes undergoing meiosis I in *D. pseudoobscura* (n=4; Figure 2.3D).

The number of sperm produced after a single round of spermatogenesis in *pse/per* hybrids (n=10) is roughly half than *D. pseudoobscura* (n=10; Figure 2.4). This difference

was significant (P<0.001) and had a large effect size (Δ=9.45). Examination of the flagellar ultrastructure in *pse/per* hybrid males reveals an identical observation of *sim/mau* hybrids. Each sperm cell possesses two sperm tails and no differentiation of the mitochondrial derivatives (Figure 2.3G), an observation not seen in *D. persimilis* sperm cells (Figure 2.3E).

Sperm of young *pse/per* hybrids (n=10) were of a single nuclei length, similar to that of parasperm found in *D. pseudoobscura* (n=5) and *D. persimilis* (n=5; Figure 2.8). Sperm of aged *pse/per* hybrids (n=10), however, differentiated into two different sperm nuclei lengths, herein referred to as "short" and "long" sperm (Figure 2.5, 2.9). In comparing sperm head length of young *pse/per* hybrids to aged *pse/per* hybrids, a given *pse/per* individual would produce sperm nuclei that is longer than sperm nuclei of a young *pse/per* hybrid ("long" sperm), a difference that is significant (P<0.05) and with a large effect size (Δ=1.01; Figure 2.5). A single *pse/per* individual would also produce sperm nuclei that is shorter than sperm nuclei of young *pse/per* hybrid ("short" sperm), a difference that is also significant (P<0.001) and with a large effect size (Δ=8.01; Figure 2.5). The "short" sperm of aged *pse/per* are shorter than parasperm of *D. persimilis* and *D. pseudoobscura,* a difference that is significant (p=0.00741 and p=0.0101, respectively) and with a large effect size (Δ=2.07 and Δ=1.92, respectively; Figure 2.9). The "long" sperm of aged *pse/per* are shorter than eusperm of *D. persimilis* and *D. pseudoobscura,* (P<0.001) and with a large effect size (Δ=2.75 and Δ=6.47, respectively; Figure 2.9).

apical tip of testis

primary spermatocyte

sperm bundle

A

B

C

D

E

F

G

H
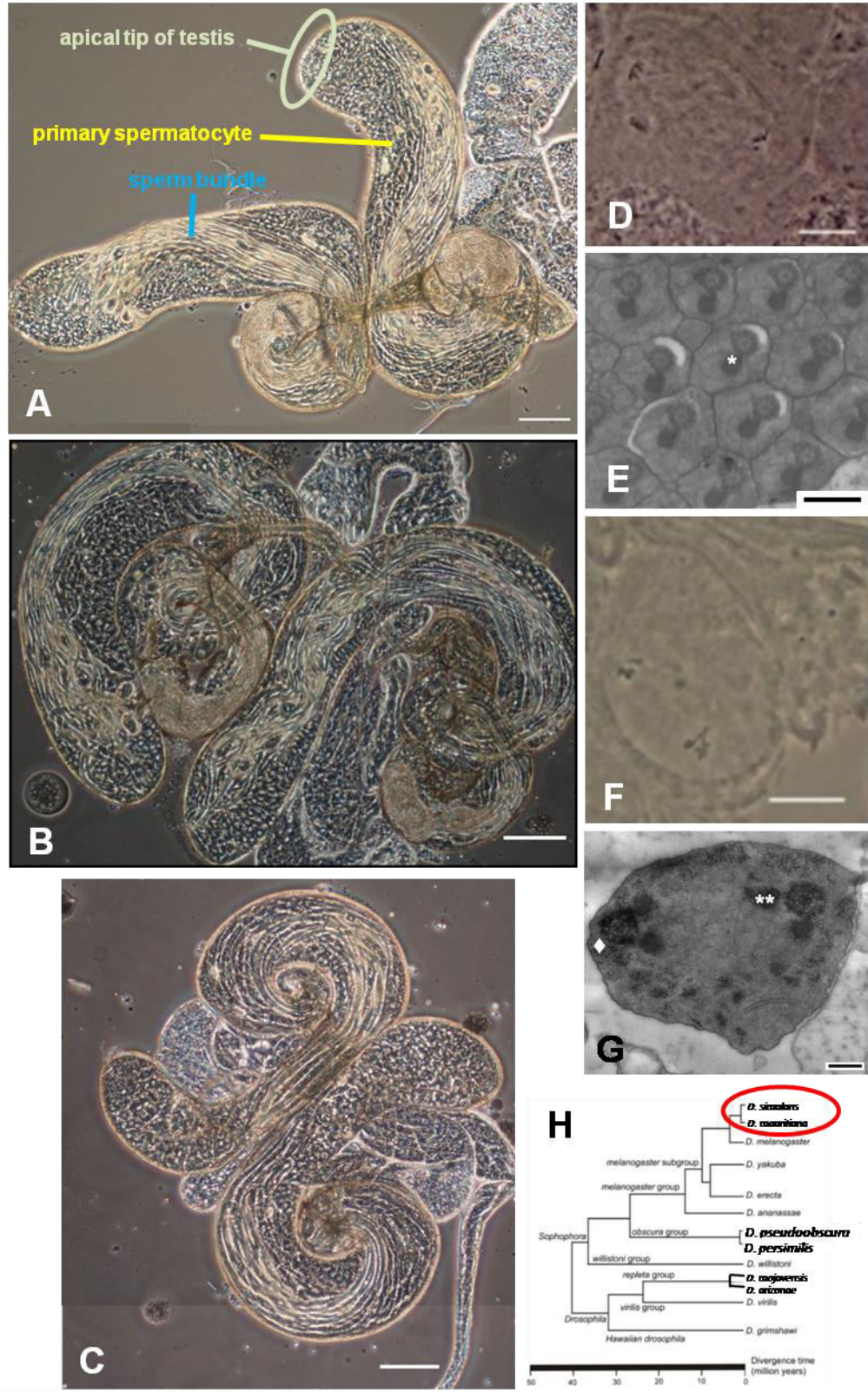
Figure 2.1: *D. simulans*/*D. mauritiana* sterile hybrid males exhibit abnormal testes morphology, improper chromosomal segregation during meiosis, and possess two-tailed sperm cells. Phase contrast images of gross testes morphology of parental species, *D. simulans* (A), *D. mauritiana* (B), and their interspecific hybrid (C). Images were taken at 10x objective and scale bar represents 0.25mm. Phase contrast images were taken of primary spermatocytes undergoing meiosis I in *D. simulans* (D), and the interspecific hybrids of *D. simulans* and *D. mauritiana* (F). Images were taken at 100x objective and the scale bar represents 0.025mm. Transmission electron microscopy (TEM) images of *D. simulans* sperm cells (E) and one sperm cell of the interspecific hybrids of *D. simulans* and *D. mauritiana* (G). Image was taken at 10500x magnification and scale bar that represents 500nm (E) or at 46000x magnification and scale bar represents 100nm (G). Phylogenetic tree of the *Drosophila genus.* Red circle highlights the location of this interspecies pair along the tree. Modified from http://rana.lbl.gov/drosophila (H). Single asterisk represents the major mitochondrial derivatives. Double asterisk represents one undifferentiated mitochondrial derivative. White diamond represents one of two sperm tails in a single sperm nucleus. Panel A outlines important structures/cells within *Drosophila* testes for this study.
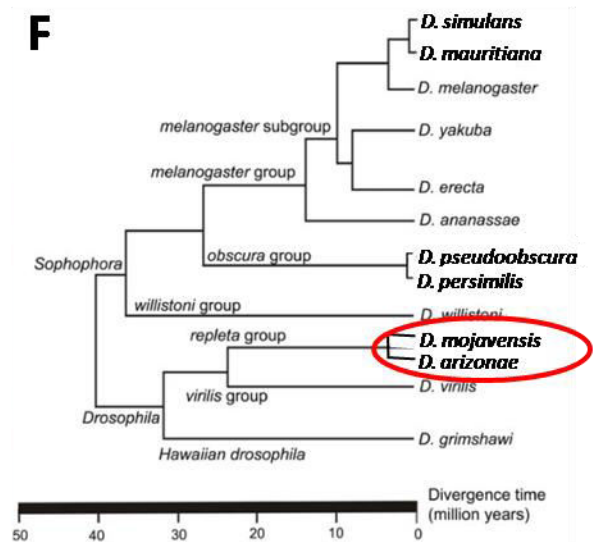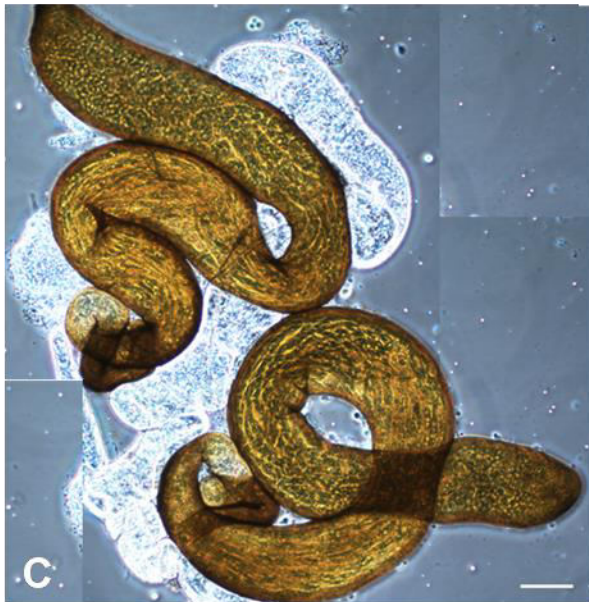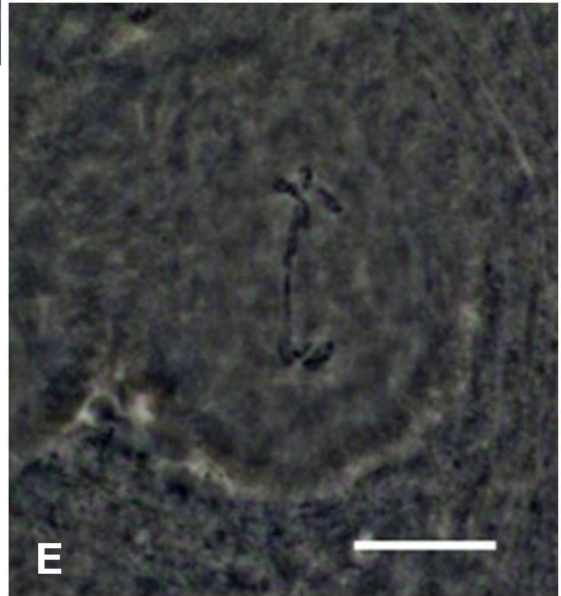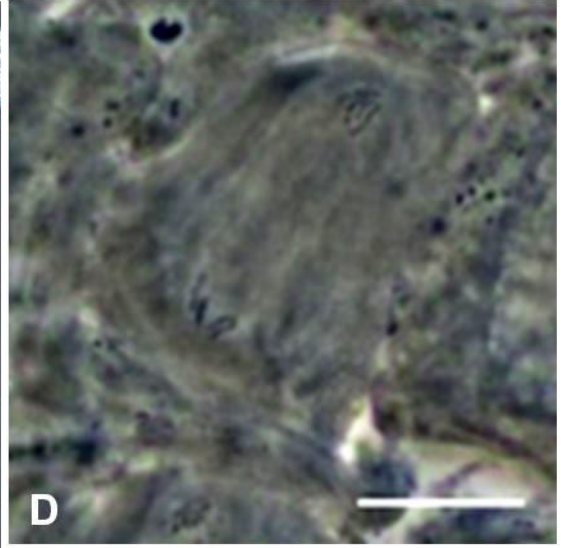
Figure 2.2: *D. mojavensis/D. arizonae* sterile hybrid males exhibit abnormal testes morphology and improper chromosomal segregation during meiosis. Phase contrast images of gross testes morphology of parental species, *D. arizonae* (A), *D. mojavensis* (B), and their interspecific hybrid (C). Images were taken at 10x objective and scale bar represents 0.25mm. Phase contrast images were taken of primary spermatocytes undergoing meiosis I in *D. arizonae* (D), and the interspecific hybrid of *D. arizonae* and *D. mojavensis* (E). Images were taken at 100x objective and the scale bar represents 0.025mm. Phylogenetic tree of the *Drosophila genus.* Red circle highlights the location of this interspecies pair along the tree. Modified from http://rana.lbl.gov/drosophila (F).
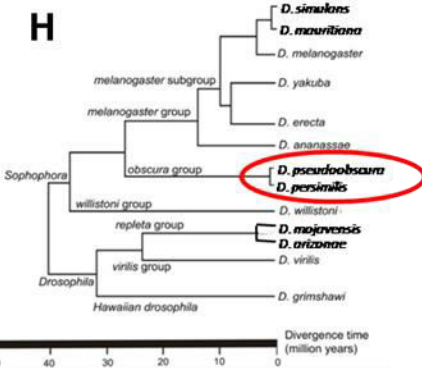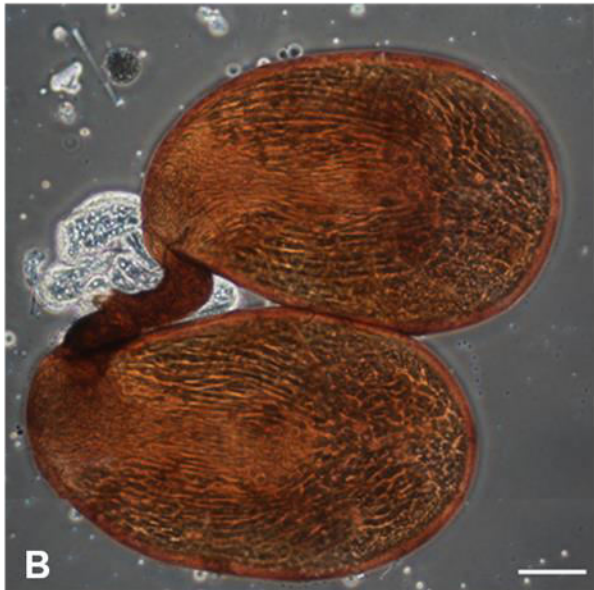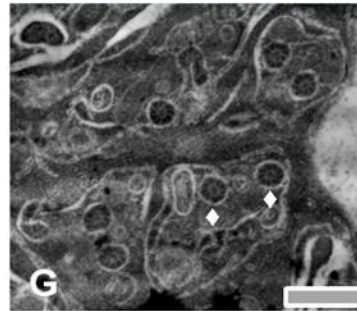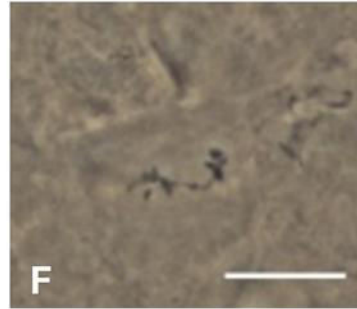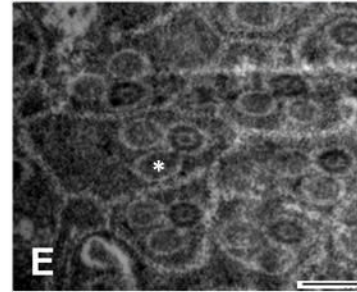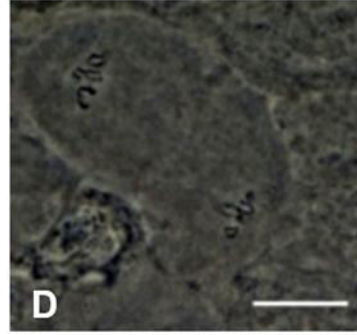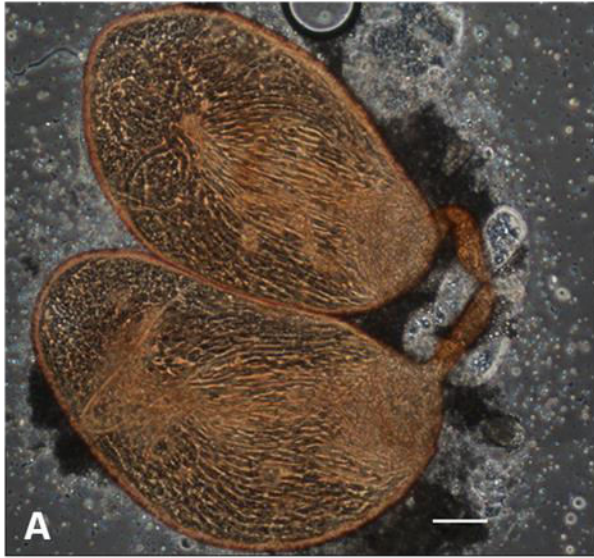
Figure 2.3: *D. pseudoobscura/D. persimilis* sterile hybrid males exhibit abnormal testes morphology, improper chromosomal segregation during meiosis, and possess two-tailed sperm cells. Phase contrast images of gross testes morphology of parental species, *D. pseudoobscura* (A)*, D. persimilis* (B), and their interspecific hybrid (C). Images were taken at 10x objective and scale bar represents 0.25mm. Phase contrast images were taken of primary spermatocytes undergoing meiosis I in *D. pseudoobscura* (D), and the interspecific hybrid of *D. pseudoobscura* and *D. persimilis* (F). Both images were taken at 100x objective and the scale bar represents 0.025mm. Transmission electron microscopy (TEM) images of *D. persimilis* sperm cells (E) and one sperm cell of the interspecific hybrids of *D. pseudoobscura* and *D. persimilis* (G). Image of *D. persimilis* sperm cells were taken at 25000x magnification and scale bar represents 500nm (E), image of interspecific hybrid sperm cell was taken at 25000x magnification and scale bar represents 500nm (G). Phylogenetic tree of the *Drosophila genus.* Red circle highlights the location of this interspecies pair along the tree. Modified from http://rana.lbl.gov/drosophila (H). Asterisk represents sperm mitochondria. White diamonds represents two sperm tails within one sperm nuclei.
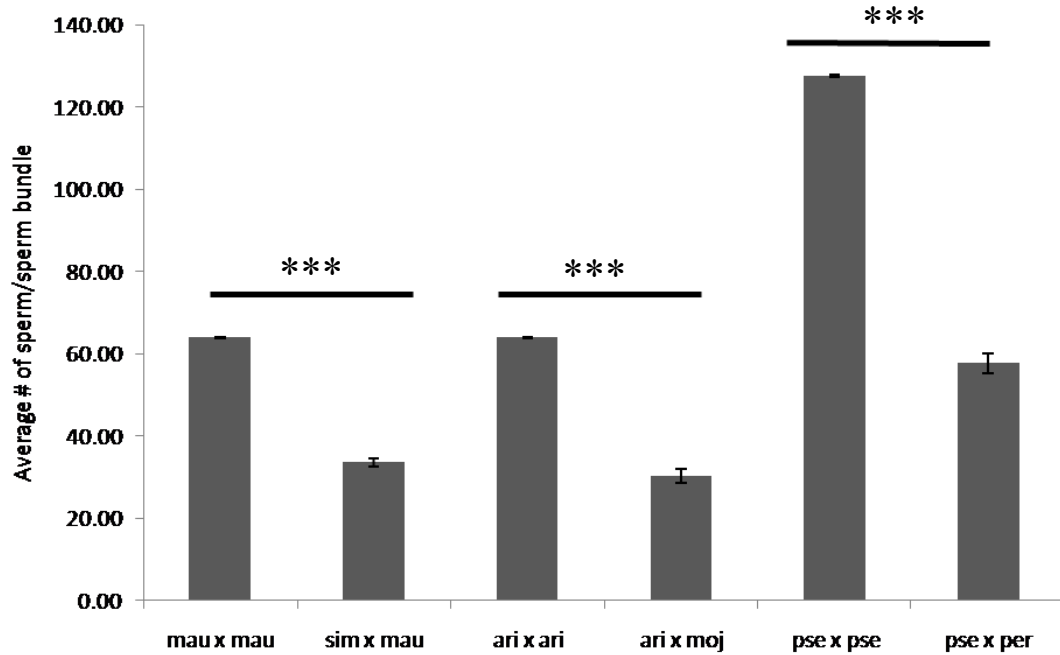
Figure 2.4: Interspecies hybrid males roughly half the number of sperm per sperm bundle than parental species males. Significantly fewer sperm ($p < 0.0001$ for all comparisons) were produced after one round of spermatogenesis (represented in the number of sperm produced within a sperm bundle) for pure species males of *D. mauritiana* (*mau*) compared to interspecies hybrids (*sim/mau*), pure species *D. arizonae* (*ari*) compared to interspecies hybrids (*ari/moj*), and pure species *D. pseudoobscura* (*pse*) compared to interspecies hybrids (*pse/per*). Error bars represent standard error.
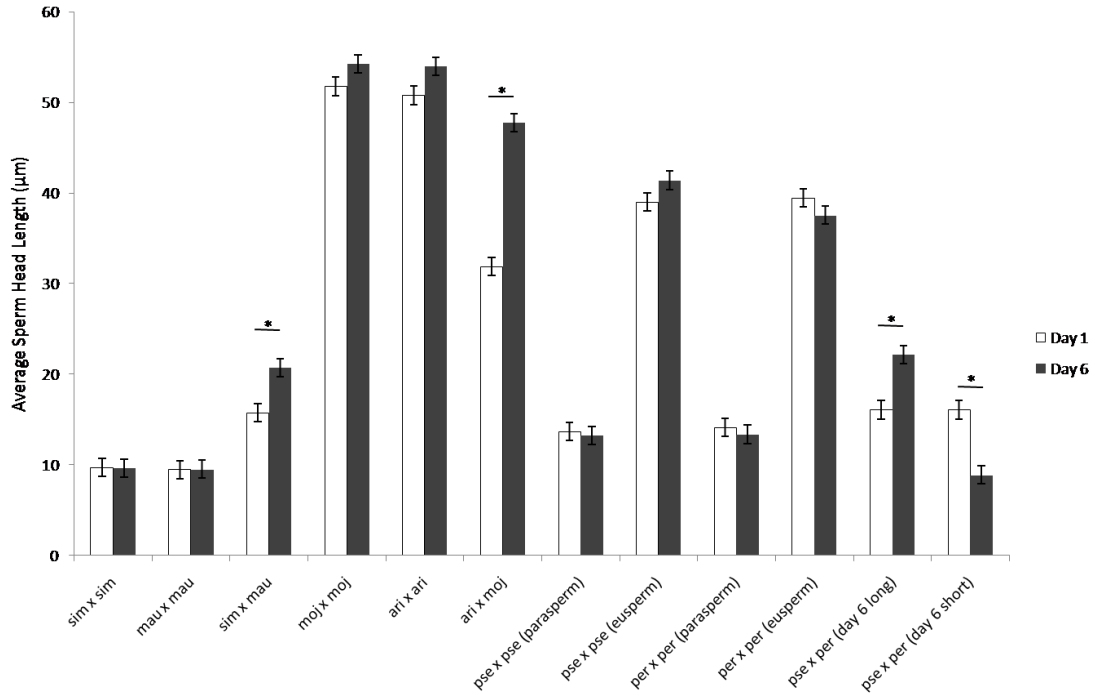
Figure 2.5: The sperm nuclei of interspecies hybrid males de-condenses with age, while similarly aged parental species males do not.Sperm head length, measured in μm, was compared between old and young males produced by intraspecific crosses (*sim/sim;mau/mau; moj/moj; ari/ari; pse/pse; per/per*)and interspecific crosses (*sim/mau*; *ari/moj;pse/per*). Sperm head length for aged (6 days old) sterile hybrid males were significantly longer than young (1 day old) sterile hybrid males ($P<0.05$). Error bars represent standard error. Histographs of the distribution of individual sperm nulei for each species and their interspecies crosses can be found in Appendix B.
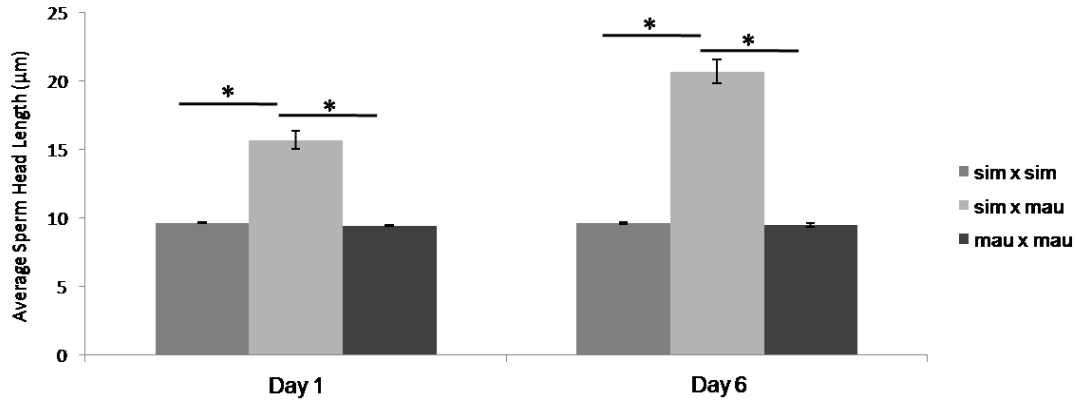
Figure 2.6: The sperm nuclei of interspecies hybrid males are longer than parental species males, for both Day 1 and Day 6 old males. Sperm head length, measured in μm, was compared between young males (Day 1) produced by intraspecific crosses (*sim/sim; mau/mau*) and interspecific crosses (*sim/mau*). The same measurement was taken for older males (Day 6). Sperm head length of interspecies sterile hybrid males was significantly longer than parental males for both old and young males (P<0.05). Error bars represent standard error.
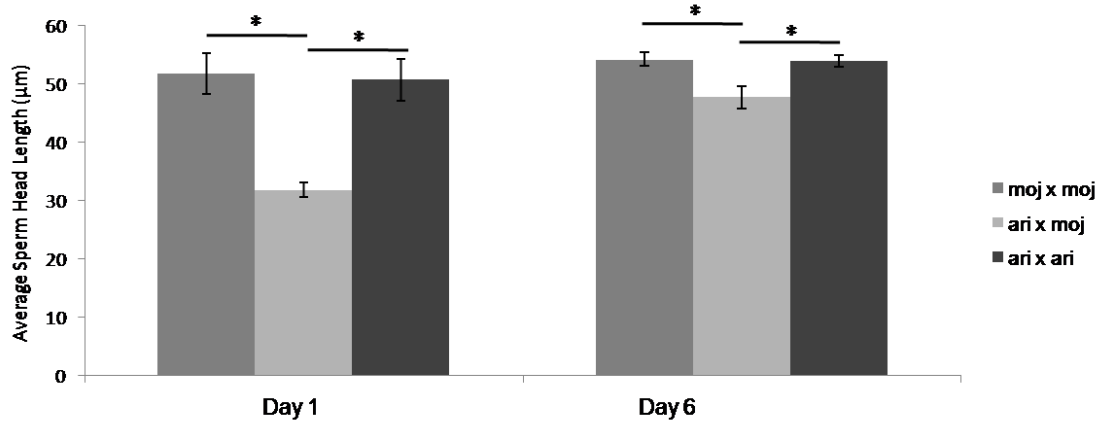
Figure 2.7: The sperm nuclei of interspecies hybrid males are shorter than parental species males, for both Day 1 and Day 6 old males. Sperm head length, measured in μm, was compared between young males (Day 1) produced by intraspecific crosses (*moj/moj; ari/ari*) and interspecific crosses (*ari/moj*). The same measurement was taken for older males (Day 6). Sperm head length of interspecies sterile hybrid males was significantly shorter than parental males for both old and young males (P<0.05). Error bars represent standard error.
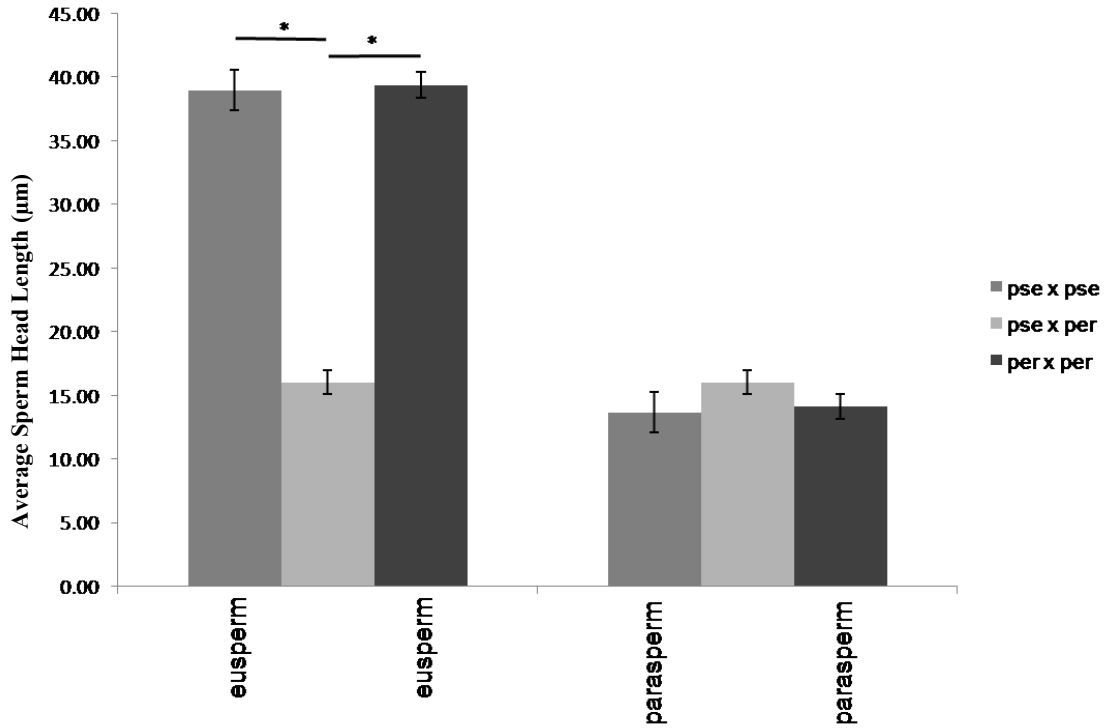
Figure 2.8: The sperm nuclei of young (Day 1) interspecies hybrid males are shorter than eusperm of young parental species males. Sperm head length, measured in μm, was compared between young males produced from intraspecific crosses (*pse/pse; per/per*) and interspecific crosses (*pse/per*) for both sperm morphs (eusperm and parasperm). Sperm head length of interspecies sterile hybrid males was significantly shorter than parental males that areproducing eusperm (P<0.05). There was no significant difference between interspecific hybrid males and parental males that are producing parasperm. Error bars represent standard error.
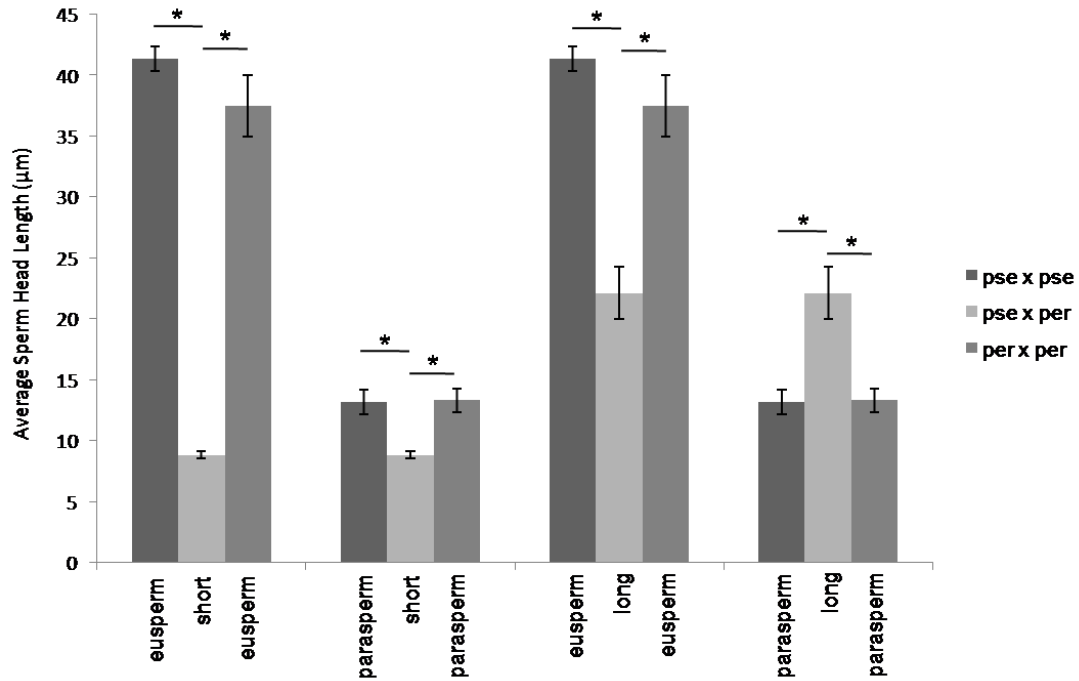
Figure 2.9: The sperm nuclei of older (Day 6) interspecies hybrid males produce two sperm morphs that are not the same sperm head length as eusperm or parasperm. Sperm head length, measured in μm, was compared between older males produced from intraspecific crosses (*pse/pse; per/per*) and interspecific crosses (*pse/per*) for both sperm morphs (eusperm and parasperm). Interspecific sterile hybrid males produced sperm with significantly different sperm head lengths than either sperm morph (P<0.05). Error bars represent standard error.

## 2.4 Discussion

It would be expected that if randomly arising mutations cause sterility in hybrid males, then unique spermatogenic failures should be seen in different interspecific hybrids (Bateson, 1909; Dobzhansky, 1936; Muller, 1940). Here, I see a common trend in testes morphology across the three interspecies pairs examined in this study. First, sperm bundles within testes of sterile hybrid males are disorganized. Second, mitochondrial derivatives are not formed and a single sperm nucleus contains two sperm tails, as seen in previous studies (Kulathinal and Singh, 1998; Hardy et al., 2011). Sterile hybrid males produced between *D. persimilis* and *D. pseudoobscura* exhibits a more severe phenotype than all other interspecies crosses studied, wherein the level of disorganization of sperm bundles is greater (Figure 2.3). Individual sperm bundles are difficult to identify in sterile hybrid males and bundles appear shorter than sperm bundles from parental species. This may be a by-product of both species possessing two different sperm morphs (Snook et al., 1994), whose spermatogenic pathways may differ from each other, or which may arise from a more complex process of spermatogenesis.

It's important to note that the length of each sperm bundle was not determined for any interspecies hybrids and should be further studied to determine if sperm cells of hybrid sterile males are truncated. For interspecific hybrids that produce sperm, I did not observe defects in spermatogenesis prior to meiosis (data not shown), a finding also noted in interspecific hybrids within the *simulans* complex (Kulathinal and Singh, 1998). Evidence of meiotic defects, however, are seen when examining chromosome composition between dividing primary spermatocytes. The observation of unequal division of chromosomes between daughter cells of meiosis I suggest possible errors in the segregation machinery during anaphase I. Furthermore, chromosome bridges have been noted in some meiotically dividing cells of two interspecies hybrids (Figure 2.2, 2.3). Chromosome bridges have been noted in interspecific hybrids in plants and been speculated to arise and cause disruption due to difference in chromosomal structures (McClintock, 1933; Beasley, 1941). Chromosome bridges have been linked to mitotic mis-segregation in hybrid inviability between two species of *Drosophila* (Ferree and Barbash, 2009). Here, improper chromatin separation is induced by divergence of a non-

coding region between *D. melanogaster* and *D. simulans,* leading to mitotic errors during early embryogenesis. Mitotic defects can be observed during anaphase, wherein the lagging chromatin, consisting of a 359-base pair repeat region, creates a chromosome bridge between the dividing chromosomes (Ferree and Barbash, 2009). Although the findings of Ferree and Barbash (2009) are exclusive to mitosis and hybrid inviability, a similar explanation may be used in the findings observed in interspecific sterile hybrid males examined in this study.

One possible scenario for mis-segregation of chromosomes during meiosis I in interspecific sterile hybrid males may be due to centromeric divergence. The machinery necessary for meiotic segregation is recruited by the centromere (Dernburg et al., 1996), a rapidly evolving region that consists of heterochromatin (Csink and Henikoff, 1998). The proteins that associate with the centromere also exhibit rapid co-evolution, establishing a unique sequence specific system between centromeres and their proteins within a given species (Malik and Henikoff, 2001; Brideau et al., 2006). Interspecific sterile hybrid males may suffer from meiotic defects as a result from improper assembly of the segregation machinery during anaphase due to divergence centromeric sequences. Henikoff et al. (2001) proposes that meiotic drive may play a role in centromeric evolution, as selfish centromeres drive the rapid divergence of centeromeric sequences. This theory, however, has been refuted since centromeric evolution should lead to interspecific hybrid female sterility (Coyne and Orr, 2004). As such, further examination of the sequences involved in the chromosomal bridge observed in this study, and what chromosomes are failing to separate, may provide insight into how chromosomes are mis-segregating during meiosis. Attempts to identify the chromosomes involved in mis-segregation have been performed by labelling sequence using *F*luorescent *in-s*itu *h*ybridization (FISH), however, attempts were unsuccessful. Finally, it's important to note that with a relatively small sample size for each parental species and interspecific hybrid (n<8), it is difficult to conclude whether bridging chromosomes are seen in all interspecies hybrids in this interspecies pair.

Overall, it appears meiotic errors may be cause for sterility across all three interspecies hybrids examined in this study. A by-product of mis-segregation of chromosomes may

also account for the reduction of sperm produced, as approximately half the number of sperm are generated after spermatogenesis in all sterile hybrid males examined (Figure 2.4). If chromosomes are unable to properly segregate, sperm cells may not be capable of properly individualizing during the process of spermiogenesis. Sperm cell head morphology has been further explored and will be discussed in Chapter 5. Regardless, chromosome mis-segregation and reduction to half the number of sperm produced after spermatogenesis across multiple species pairs have never been reported previously, yet this widespread observation suggests a common mechanism for hybrid sterility and Haldane's rule in *Drosophila.*

The most obvious cause of this disorganization may lie in the stages that occur after meiosis, collectively known as spermiogenesis. Sperm-producing hybrids possess an underdeveloped axonemal complex (Kulathinal and Singh, 1998), which is the cytoskeletal structure for sperm tails, and may experience postmeiotic defects. Proper packaging of the sperm head plays a pivotal role in sperm motility, protecting the DNA, establishing sperm head hydrodynamic morphology and ensuring successful fertilization (Bianchi et al., 1993; Agarwal and Said, 2003; Aoki et al., 2005). In addition, a fully functional tail is required for proper motility of the sperm cell, and thus can affect a sperm's ability to fertilize (Tokuyasu, 1972). In this study, two tails were seen within each sperm cell for interspecific hybrids within the *simulans* complex and the *D. pseudoobscura* subgroup. This suggests that the cross-sectional analysis of the each single cell may in fact be two sperm cells joined together, a possible by-product of the non-disjunction seen during meiosis. Furthermore, hybrids of these interspecific crosses do not have distinct mitochondrial derivatives (major and minor). Whether this lack of mitochondrial differentiation affects mitochondrial function has yet to be determined, but could account for the lack of motility in hybrid sperm (addressed in Chapter 3).

Interspecific hybrids of *D. arizonae* and *D. mojavensis* also have similar disorganization of sperm bundles, but may or may not have two-tailed sperm, as the images were not conclusive (Hardy et al., 2011). Hybrids produced from female *D. mojavensis* mated to male *D. arizonae* do not appear to have paired sperm tail. Conversely, hybrid males produced from the reciprocal cross appear to exhibit a more disorganized sperm bundle

and sperm cells may possess two sperm tails; however, it was difficult to differentiate between sperm cells accurately (Hardy et al., 2011). Regardless, some of the findings of Hardy et al. (2011) are similar to the observations made in this study. When female *D. arizonae* females are mated to *D. mojavensis* males, roughly half the number of sperm are produced after spermatogenesis in sterile hybrid males, compared to the parental species (Hardy et al., 2011; Figure 2.4). Furthermore, mitochondria within each sperm bundle did not differentiate into the major or minor mitochondrial derivatives, a finding similar to both *pse/per* and *sim/mau* hybrids (Hardy et al., 2011; Figure 2.1, 2.3). Although sterile hybrids produced by female *D. mojavensis* mated to male *D. arizonae* did not exhibit two-tailed sperm cells or half the number of sperm produced (Hardy et al., 2011), it has been previously shown that maternity plays a role in different sterility phenotypes within *Drosophila* (Kulathinal and Singh, 1998). When female *D. mauritiana* are mated to *D. simulans* (or *D. sechellia*), sterile hybrid males do not produce sperm and spermatogenesis appears to stop prior to meiosis (Kulathinal and Singh, 1998). Both studies suggest that maternal factors (either genetic or cellular) play a role at which stage of spermatogenesis is affected in sterile hybrids. Although this not the common trend seen in other interspecific crosses in *Drosophila* (e.g. *pse/per* hybrids; Figure 2.3 or hybrids of *D. simulans* and *D. sechellia*; Kulathinal and Singh, 1998), it's important to note that Haldane's rule may not specifically target one stage of spermatogenesis and slight phenotypic variations may be observed.

Sperm nuclei of *sim/mau* hybrids did appear less condensed than that of the parental species, suggesting possible abnormalities in DNA packaging and protamine expression, the latter of which has been previously shown in *D. simulans* and *D. mauritiana* hybrids (Moehring et al., 2007). Sperm nuclei from *ari/moj* hybrids were shorter than that of the parental species, even though aged males (day 6) possessed longer nuclei than young males, suggesting nuclei of hybrids are more condensed than their parental sperm nuclei. A similar phenomenon was seen with young hybrid male (day 1) sperm produced from *pse/per*. Nuclei length was similar to the length of parasperm found in both parental species, but upon aging, the nuclei would either de-condense (long sperm) or condense further (short sperm). Neither long sperm nor short sperm length were similar to either sperm morphs of *D. pseudoobscura* and *D. persimilis*. As with the increased

abnormalities in gross testes morphology, it is possible that these more extreme results seen within hybrids of *pse/per* could be due the different sperm morphs produced in both parental species (Snook et al., 1994). It is also possible that young interspecific hybrid males are only producing parasperm, but later produce two sperm morphs that not able to properly package into parasperm or eusperm. Further inquiry to this question may provide insight as to the abnormalities in DNA packaging in hybrids, although it may be possible these downstream effects are a by-product of errors occurring earlier in spermatogenesis.

Overall, several trends are observed in many interspecific sterile hybrid males within *Drosophila.* First, sperm bundles are not properly organized within testes of sterile hybrid males. Second, meiotic errors are apparent for most hybrids examined in this study, as sperm cells exhibit non-disjunction after meiosis I, roughly half the number of sperm a produced after spermatogenesis, and each cell possesses two sperm tails. As previously speculated, meiotic drive may be the general cause for meiotic errors observed in sterile hybrid males. Within a population, a selfish driver and its suppressor co-evolve, leading to an interaction that is compatible within the given population. Hybridization, due to interspecific mating, may break-up the interaction, leading to defects within the hybrid. How those defects manifest may involve non-coding sequences (leading to mis-segregation of chromosomes during meiosis) or apoptosis of cells not possessing the driver element (leading to half the number of sperm produced). Examination of the sequences leading to meiotic errors (perhaps by isolation of chromosome bridges) could provide further insight.

The general trends noted in this study potentially contradict the BDM model, as it predicts failures within sterile hybrid males to be inconsistent and random, as the mutations that would lead to genetic incompatibility are random (Bateson, 1909; Dobzhansky, 1934; Muller, 1940). However, the results of this study consistently point to similar failures during spermatogenesis across many interspecies pairs in *Drosophila.* Thus, a common stage at which spermatogenesis is failing within sterile hybrids may suggest two possibilities. First, random genetic changes may arise, causing divergence between populations and lead to hybrid sterility. How hybrid sterility manifests, however,

is similar across different interspecific hybrid males, such that a particular stage of spermatogenesis is prone to failure. In other words, common trends when studying hybrid sterility in males may exhibit cellular consistency, but the genetic underpinnings of those consistencies are random. Second, the genetic basis for hybrid sterility is not random and the genetic factors that are involved in a particular stage of spermatogenesis are more likely to diverge, leading to errors and hybrid sterility.

Taken together, the observation of non-disjunction, the presence of chromosomal bridges in some cases, and the production of half the number of sperm per sperm bundle suggests failures occurring during the first meiotic event, rather than the second, across all three species pairs. If this is indeed a common trend, then errors during meiosis I may be the key to understanding the nature of hybrid breakdown, the evolution of hybrid sterility, and Haldane's rule.

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# Chapter 3

# 3   Mitochondrial activity and alive *versus* dead sperm in sterile hybrid males in *Drosophila.*

Sterile hybrid males in a variety of interspecies crosses in *Drosophila* produce immotile sperm with mitochondria that do not differentiate into the major and minor derivatives, suggesting a lack of function. Separately, it is unknown if sperm from sterile hybrid males are dead or alive. In this study, I used three different interspecific *Drosophila* crosses to determine the following in sterile hybrid males: are sperm dead or alive, and is a lack mitochondrial activity associated with immotility. I found that the sperm of sterile hybrid males are always alive and have entirely active mitochondria.

## 3.1   Introduction

In *Drosophila melanogaster,* sperm morphological abnormalities within sterile males have been extensively studied (Hardy et al., 1981; Kiefer, 1996; Wakimoto et al., 2004). A misshapen sperm head, improper packaging of DNA within the sperm head, dysfunctional mitochondria that affect sperm motility, and the production of dead sperm are associated with reduced male fertility. As such, any one of these factors may contribute to interspecies hybrid male sterility.

In insects, the presence of live or dead sperm has been studied in the context of sperm competition (Hunter and Birkhead, 2002; Garcia-Gonzalez and Simmons, 2005), but the implications for hybrid sterile males has not been considered. Conceptually, a reduction in live sperm will have an effect on the concentration of sperm in the ejaculate that can fertilize an egg (Galvani and Johnstone, 1998; Guzick et al., 2001; Pacey, 2009). Therefore, in sterile hybrid males, it is plausible that sterility is due to complete loss of live sperm.

Mitochondria produce energy to drive flagellar movement and active mitochondria can be used as an indicator of a sperm tail's ability to move (Ruiz-Pesini et al., 1998).

Furthermore, assaying for sperm tail movement, using light microscopy, has been a commonly used measurement for mapping hybrid sterility genes in previous studies and is assumed to be directly correlated with the underlying genetic basis of hybrid sterility (Perez et al., 1993; Perez and Wu, 1995; Masly et al., 2006; Phadnis and Orr, 2009). Upon closer inspection of sperm tails at the ultrastructural level, it was noted that the mitochondria did not properly differentiate into the major and minor derivatives (Chapter 2). To date, the function of each derivative is unclear, but it is known that most of the contents within the minor mitochondrial derivative are removed during sperm individualization (Tokuyasu et al., 1972), suggesting it may not serve a function in sperm tail motility. As such, it is possible that sterile hybrid males possibly have inactive mitochondria, due to the failure to fully form both mitochondrial derivatives, leading to immotile sperm.

As outlined above, in Chapter 2, sterile hybrid males produce roughly half the number of sperm, and mitochondria do not differentiate into the major and minor derivatives. How this contributes to hybrid male sterility in *Drosophila* requires further study. Since hybrid males produce half the number of sperm from parental species, sperm cells may be undergoing apoptosis, and thus males produce dead sperm. In addition, the presence of two non-differentiated mitochondria in sterile hybrid males may lead to dysfunctional mitochondrial activity. As such, testing for active mitochondria allows me to assess whether undifferentiated mitochondrial derivatives are non-functional, thus leading to immotile sperm tails in sterile hybrid males. Thus, the purpose of this study was to identify alive *versus* dead sperm and mitochondrial activity within hybrid sterile males in three interspecies crosses within *Drosophila*. Using a Mitotracker® Red CMXRos stain that measures mitochondrial membrane potential, sperm tails of all three interspecies crosses did not appear to have reduced mitochondrial membrane potential. Using a live/dead sperm assay, hybrid males from all three interspecies crosses were living. This suggests that sterility is not a result of inactive mitochondria, nor is it a result of sperm inviability.

## 3.2 Materials and methods

### 3.2.1 Fly husbandry and interspecies crosses

All *Drosophila* stocks were obtained from the *Drosophila* Species Stock Center (San Diego, CA, USA). All flies, with the exception of those from the *repleta* group were maintained on standard recipe cornmeal media (Bloomington Drosophila Stock Center recipe). *Drosophila arizonae* and *Drosophila mojavensis* were maintained on banana media (Markow and O'Grady, 2006). Flies were housed at 24˚C on a 14:10 hour light:dark cycle at 75% humidity. Interspecies crosses were set up for three different groups within the genus *Drosophila.* Approximately 5 virgin female *Drosophila simulans* were mated to 5 virgin male *Drosophila mauritiana.* Similarly, approximately 5 virgin female *Drosophila persimilis* were mated to 5 virgin male *Drosophila pseudoobscura.* Lastly, 5 virgin female *D. arizonae* were mated to 15 virgin male *D. mojavensis.*

### 3.2.2 Live/dead sperm assay

To assess sperm viability, I collected males from each species studied, and sterile hybrid males produced from each interspecies cross, and aged flies for one day. Testes were extracted in approximately 200µl of Testes Buffer (183m$M$ KCl, 47m$M$ NaCl, 10m$M$ Tris-HCl) then transferred to 20µl of a mixed Live/Dead solution on a siliconized cover slip. This solution contains 44µ$M$ of SYBR green and 50µ$M$ of propidium iodide (PI; Thermo Fisher Scientific). PI penetrates damaged and inactive cell membranes, staining nuclear material red, while SYBR green stains DNA green (Garner et al., 1994). This combination of nuclear stains can be used to analyze sperm cells that are alive (stains with SYBR green) or dead (stains with both SYBR green and PI) and has been previously used to detect apoptotic cells (Riccardi and Nicoletti, 2006). Testes were ripped apart in the Live/Dead solution to expel contents and were incubated at room temperature in a dark chamber for 5mins. The siliconized cover slip kept the Live/Dead mixture in a droplet form, preventing the liquid from spreading across the cover slip and drying during the incubation period. The Live/Dead solution was carefully removed using a pipette, then 20µl of Testes Buffer was added to the testes sample and washed by gently pipetting

the solution for 1 min. A second wash step was performed with fresh Testes Buffer to ensure the sample was properly washed and all contents of the Live/Dead solution were removed. Testes solution was carefully removed, 20µl of fresh Testes Buffer was dropped onto the sample, and the cover slip was mounted on a glass slide. Samples were then viewed using a fluorescent microscope. The first 30 sperm located using bright field microscopy had their sperm nuclei scored as alive *versus* dead using fluorescence. Five males from each parental species and 10 sterile hybrid males from each interspecies cross were examined. As a negative control, sperm cells from *D. simulans* were incubated in Triton X-100 for 5 mins to break up the cell membrane. These dead sperm cells were then processed and scored for viability as described above. Images were taken using a Nikon Eclipse Ci-L upright fluorescent microscopy, equipped with a DS-Fi2 colour camera (Nikon Canada). Image acquisition used Nikon Elements D software and further image processing was done using ImageJ (Schneider et al., 2012).

### 3.2.3   Sperm mitochondrial membrane potential assay: MitoTracker® CMXRos

To assess sperm mitochondrial activity, males from each species studied and sterile hybrid males from each interspecies cross were collected upon eclosion and aged for one day. Testes were extracted in 200µl of Testes Buffer (183m$M$ KCl, 47m$M$ NaCl, 10m$M$ Tris-HCl) and then transferred to 20µlof 100mM of Mitotracker® Red CMXRos (ThermoFisher Scientific) on a siliconized cover slip. Mitotracker® Red CMXRos is a mitochondrial specific stain that accumulates in signals depending on the membrane potential (Pendergrass et al., 2004). Testes were ripped apart in the Mitotracker® Red CMXRos solution to expel the contents and then incubated for 10mins at room temperature in a dark chamber. The siliconized cover slip kept the Mitotracker® Red CMXRos solution in a droplet form, preventing the liquid from spreading across the cover slip and drying during the incubation period. The Mitotracker® Red CMXRos solution was carefully removed using a pipette and 20µl of Testes Buffer was added to the testes sample and washed by gently pipetting the solution for 1 min. A second wash step was performed with fresh Testes Buffer to ensure the sample was properly washed and all contents of the Mitotracker® Red CMXRos solution was removed. Testes

solution was carefully removed and 20µl of fresh Testes Buffer was dropped onto the sample; the cover slip was then mounted on a glass slide. The first 50 sperm tails identified using bright field microscopy were assessed for membrane potential using fluorescent microscopy. Five males from each parental species and 10 sterile hybrid males from each interspecies cross were examined. As a negative control, sperm cells from *D. simulans* were incubated in acetic acid for 5mins, washed in Testes buffer 3 times, then processed and scored for mitochondrial activity as described above. Images were taken using a Nikon Eclipse Ci-L upright fluorescent microscopy, equipped with a DS-Fi2 colour camera (Nikon Canada). Image acquisition used Nikon Elements D software and further image processing was done using ImageJ (Schneider et al., 2012).

## 3.3   Results and discussion

### 3.3.1   Sperm viability in hybrid males of three interspecific crosses in *Drosophila*

*D. simulans* was used as a control to test the efficacy of the Live/Dead assay for sperm viability. It was confirmed that the Live/Dead sperm viability assay previously described is usable to test for sperm inviability (Figure 3.1 S-U). All sperm examined from males of *D. simulans* did not stain with PI and stained with only SYBR green (Figure 3.1A-C). All sperm from *D. mauritiana* also stained similarly to *D. simulans* (Figure 3.1D-F). Since no sperm exhibited red fluorescence, all sperm cells were alive. Sperm examined from hybrid sterile males also did not take up propidium iodide (PI) (Figure 3.2 A-C), indicating that sperm cells were not dead and their membrane is intact and undamaged in hybrids.

All sperm examined from males of *D. persimilis* did not take up PI but stained with SYBR green (Figure 3.1 G-I). This was also seen in *D. pseudoobscura* (Figure 3.1 J-L). As such, sperm produced are alive and not dead. Sperm examined from hybrid sterile males also did not take up propidium iodide (Figure 3.2 D-F), indicating that sperm cells were not dead and had no damaged cell membrane. All sperm examined from males of *D. arizonae* only stained with SYBR green, denoting viable sperm (Figure 3.1 M-O). Sperm of *D. mojavensis* stained similarly to *D. arizonae* (Figure 3.1 P-R). Sperm examined from

hybrid sterile males also did not stain with PI (Figure 3.2 G-I), indicating that sperm cells were viable and cell membranes were not damaged.

Sperm produced from sterile hybrid males in any of the interspecies crosses were viable with undamaged membranes. Although the cell membrane may be intact, it is still possible for the structure of the cell membrane to be different between sperm from sterile hybrids and from the respective pure species. Although no previous study has demonstrated the role of cell membrane structure on sperm fertility or motility, changes to the cell membrane could impact these traits.

As sterile hybrid males did not produce dead sperm, either the cells apoptose at an early stage of spermatogenesis and fully degrade by the stage I measured, or sperm cell apoptosis may not be an explanation for the reduction of sperm produced after spermatogenesis. Apoptosis may not be an explanation, as sterile hybrid males did not differ from the expected number of sperm tails (Chapter 2). As such, the reduction of sperm production in sterile hybrid males is likely due to sperm cells not properly separating during individualization. Thus, a single sperm may actually be paired sperm heads with two tails. This possibility will be further examined in Chapter 5.

## 3.3.2 Mitochondrial activity in hybrid males of three interspecies crosses in *Drosophila*

*D. simulans* was used as a control to test the efficacy of the Mitotracker® Red CMXRos for mitochondrial function. It was confirmed that the Mitotracker® Red CMXRos assay previously described is usable to test for a lack of mitochondrial activity (Figure 3.3 S-U).All sperm examined from males of *D. simulans* (Figure 3.3 A-C) and *D. mauritiana* (Figure 3.3 D-F) stained with Mitotracker® Red CMXRos all exhibited brightly fluorescent tails, indicating highly active mitochondria. All sperm examined from interspecies hybrid males between *D. simulans* and *D. mauritiana* also possessed brightly stained sperm tails, indicating functional mitochondria. However, I noted that cyst-like structures were present along the entire length of the tail (Figure 3.4 A-C).

All sperm tails from males of *D. persimilis* (Figure 3.3 G-I) and *D. pseudoobscura* (Figure 3.3 J-L) stained intensely with Mitotracker® Red CMXRos. Sperm tails examined from hybrid sterile males between *D. persimilis* and *D. pseudoobscura* also stained intensely (Figure 3.4 D-F). Sperm tails for parental species and their sterile hybrids possess functional mitochondria. All sperm tails examined from males of *D. arizonae* (Figure 3.3 M-O) stained intensely with Mitotracker® Red CMXRos, similarly to *D. mojavensis* (Figure 3.3 P-R), suggesting highly active mitochondria for both parental species. Sperm tails examined from interspecies hybrid males between *D. arizonae and D. mojavensis* also stained intensely, suggesting functional mitochondria (Figure 3.4 G-I). This is unexpected, as mitochondrial derivatives to not form in sterile hybrid males, rather, two similar mitochondria are present (Chapter 2). It is possible that each mitochondria are active and that immotile sperm may be a by-product of other sperm abnormalities in sterile hybrid males.

Cyst-like structures were also visible along the entire length of the sperm tail (Figure 3.4 G-I). This has not been previously characterized within sterile hybrid males but has been seen in *Drosophila* that are mutant for the gene producing the clathrin heavy chain (Chc) protein. Males with this mutation fail to fully individualize and are sterile (Fabrizio et al., 1998) but their sperm tails exhibit a "blebbing" defect. These blebs are thought to be a by-product of cytoplasm left behind during the individualization stage of spermiogenesis (Fabrizio et al., 1998). The blebbing phenotype has not been extensively examined but has been noted previously as occurring due to errors during individualization (Metzendorf and Lind, 2010).

I have concluded, based on the evidence of this study, that sterile hybrid males in *Drosophila* produce sperm that are alive and with active mitochondria. As such, I focus on the DNA organization and sperm head morphology and their association with hybrid sterility is the next chapters.
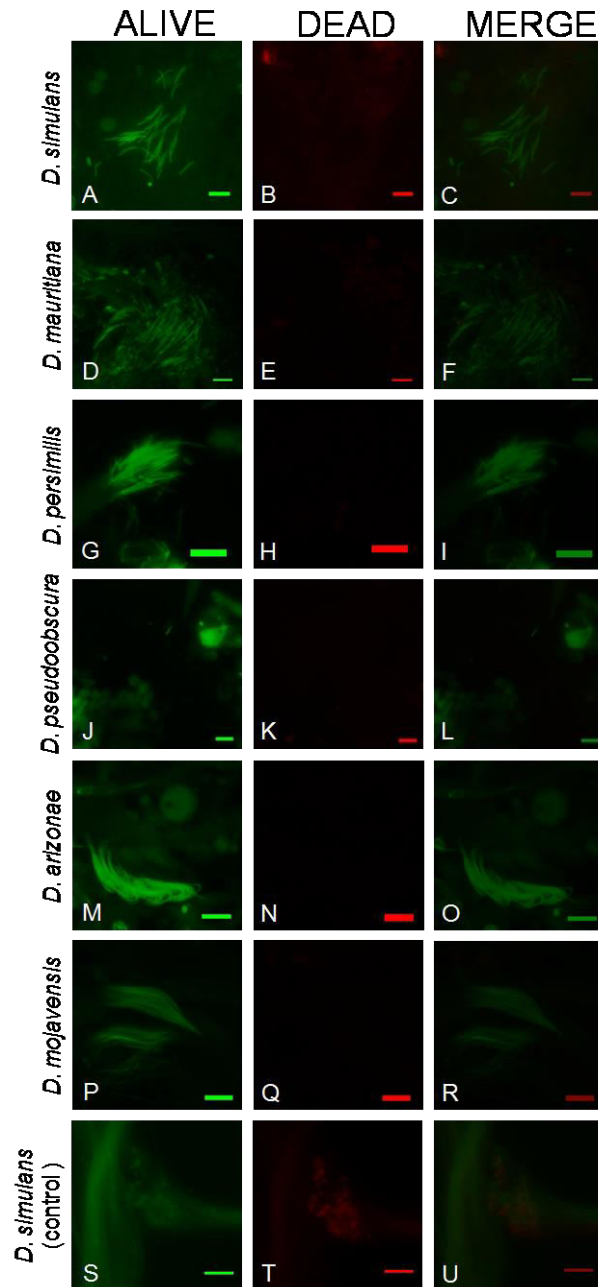
Figure 3.1: Sperm of *Drosophila* parental species contain no dead sperm within their ejaculate when stained with SYBR Green (alive sperm, green fluorescence) and propidium iodide (dead sperm, red fluorescence). Only alive sperm (green fluorescence) are found in *D. simulans* (A-C), *D. mauritiana* (D-F), *D. persimilis* (G-I), *D. pseudoobscura* (J-L), *D. arizonae* (M-O), and *D. mojavensis* (P-R). Dead sperm of *D. simulans* males (were used as a control to test the validity of the Live/Dead Sperm Assay (S-U). Scale bars represents 10μm.
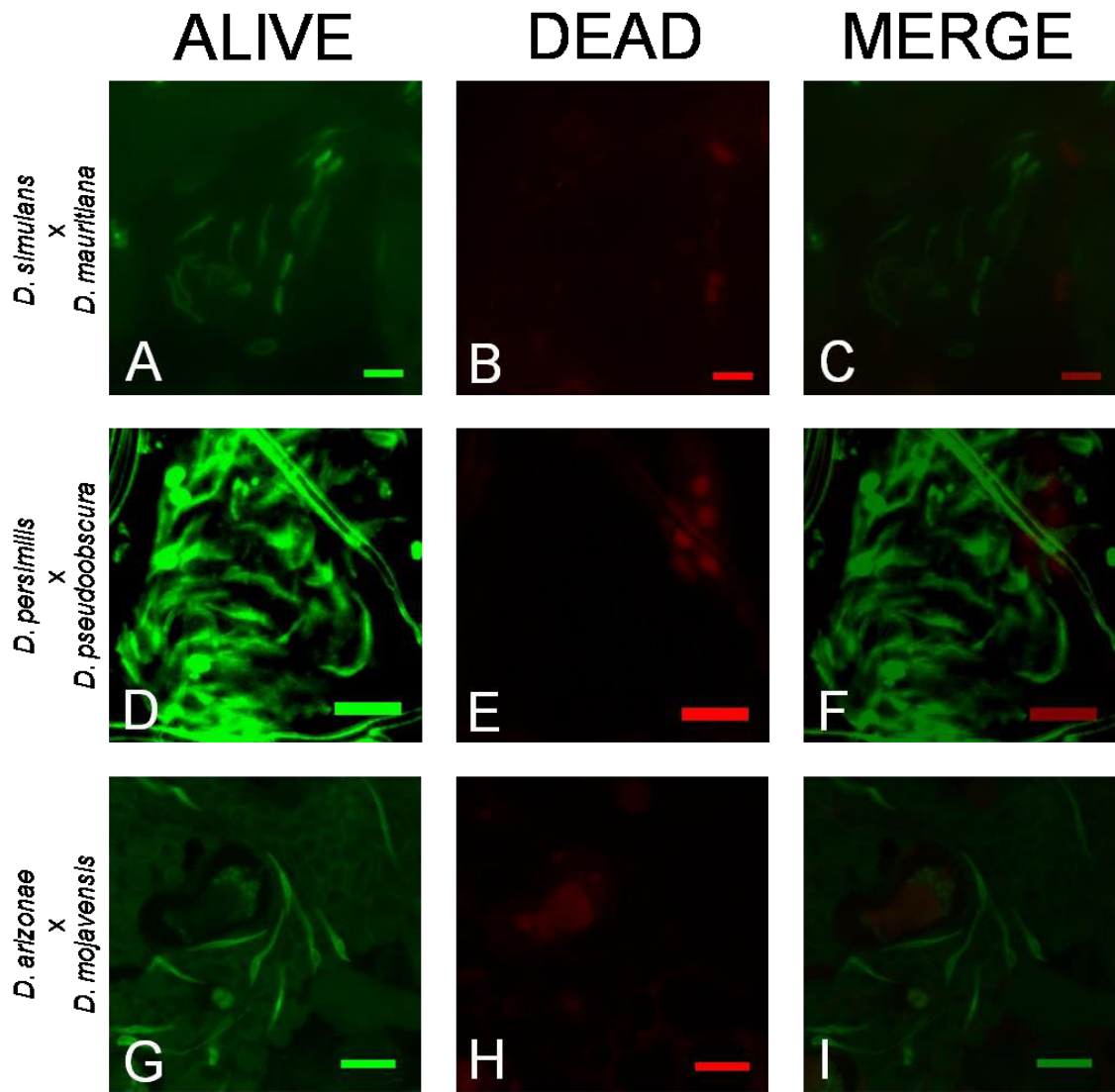
Figure 3.2: Sperm of interspecific male hybrids in *Drosophila* contain no dead sperm within their ejaculate when stained with SYBR Green (alive sperm, green fluorescence) and propidium iodide (dead sperm, red fluorescence). Only alive sperm (green fluorescence) are found in interspecific male hybrids between *D. simulans* and *D. mauritiana* (A-C), *D. persimilis* and *D. pseudoobscura* (D-F), and between *D. arizonae* and *D. mojavensis* (G-I). Scale bars represents 10μm.
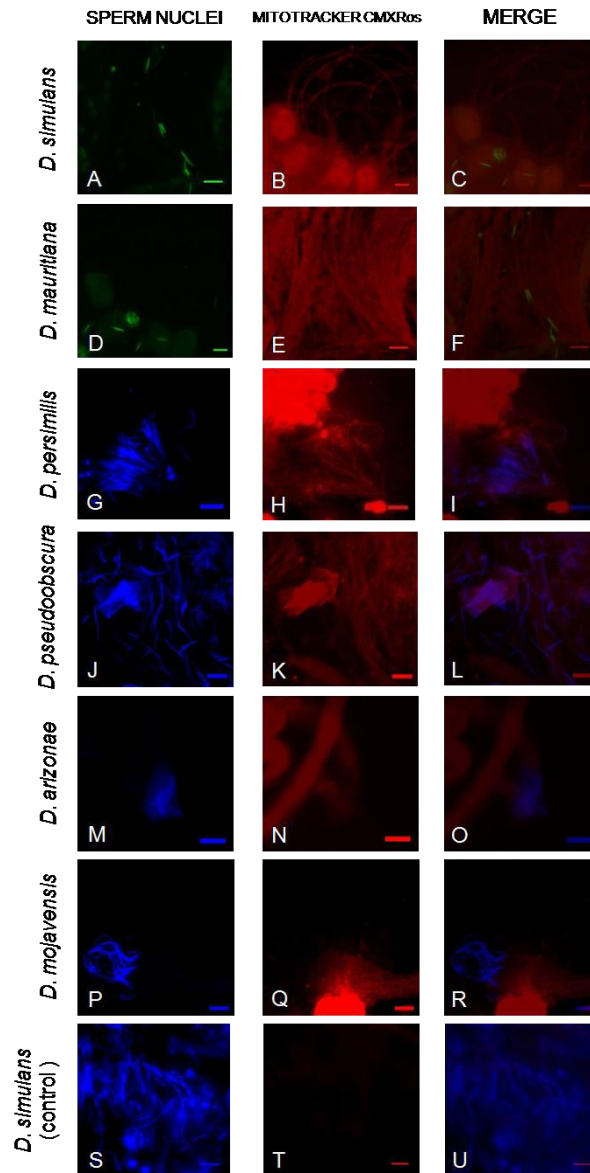
Figure 3.3: Sperm tails of *Drosophila* parental species contain active mitochondria when stained with MitoTracker® CMXRos. Active mitochondria (red fluorescence) are found along the entire length of the sperm tail in *D. simulans* (B,C), *D. mauritiana* (E,F), *D. persimilis* (H,I), *D. pseudoobscura* (K,L), *D. arizonae* (N,O), and *D. mojavensis* (Q,R). Sperm nuclei were identified by either staining with DAPI (blue fluorescence; G,J,M,P,S) or protamines where attached to GFP (green fluorescence; A,D). Non-motile sperm of *D. simulans* males were used as a control to test the validity of the MitoTracker CMXRos® Assay (S-U). Scale bars represents 10μm.
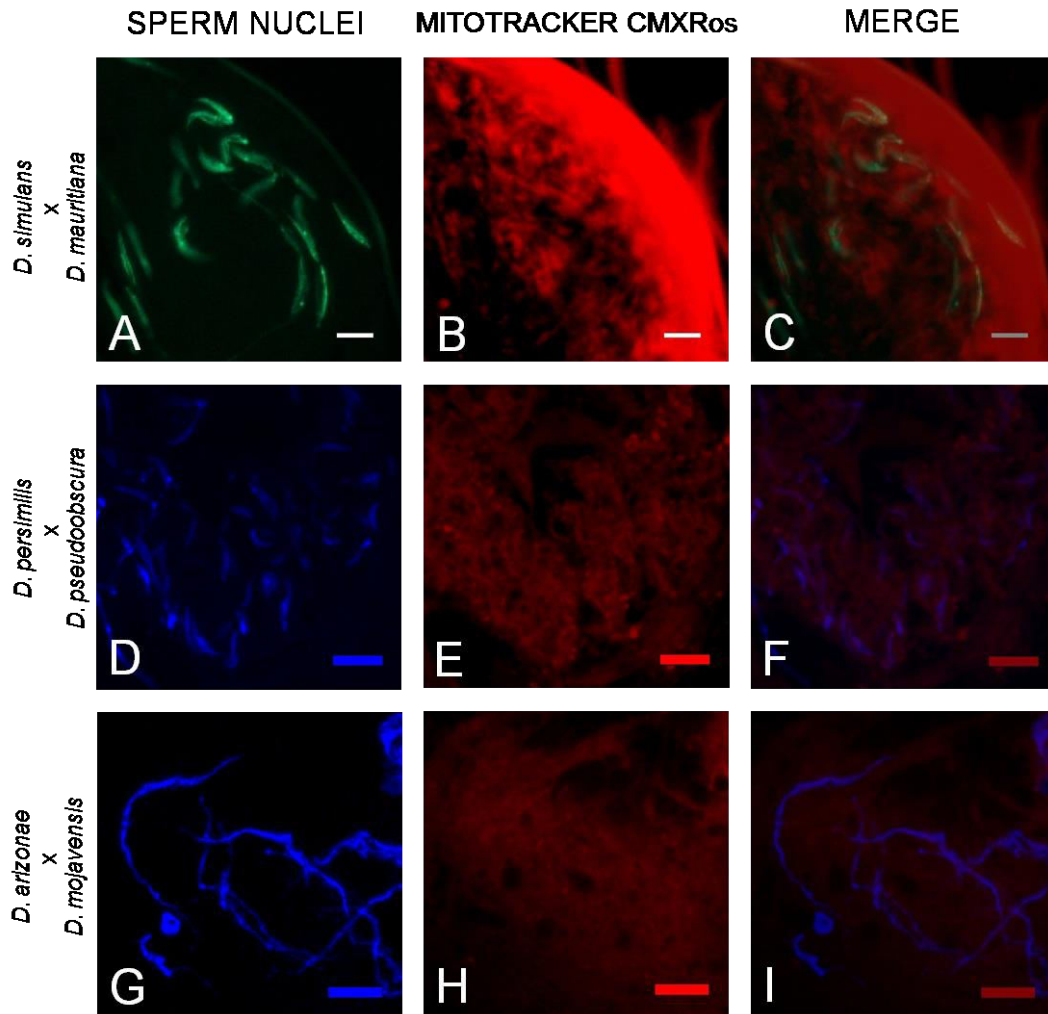
Figure 3.4: Sperm tails of interspecific male hybrids in *Drosophila* contain active mitochondria when stained with MitoTracker®CMXRos. Interspecific male hybrids between *D. simulans* and *D. mauritiana* (A-C), *D. persimilis* and *D. pseudoobscura* (D-F), and between *D. arizonae* and *D. mojavensis* (G-I). Sperm nuclei were identified by either staining with DAPI (blue fluorescence; D,G) or protamines where attached to GFP (green fluorescence; A). Scale bars represents 10μm.

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Chapter 4

# 4 Allelic expression of *Drosophila* protamines during spermatogenesis

This chapter was published in a similar form in *International Journal of Evolutionary Biology* (Kanippayoor and Moehring, 2012).

In typical somatic cells, DNA is tightly organized by histones that are necessary for its proper packaging into the nucleus. In sexually-reproducing animals, the haploid product of male meiosis must be further condensed to fit within sperm heads, thus requiring an even greater degree of packaging. This is accomplished in most organisms by replacing histones with protamines, which allows DNA to be compacted into the reduced space. In mammals, protamines are produced after meiosis is complete, and are transcribed by the single allele present in the haploid genome that is to be packaged into the sperm head. Here, I present my findings that protamine expression occurs from both alleles in diploid cells, rather than haploid cells, in two species of *Drosophila*. Diploid expression of protamines is also seen in sterile hybrid males produced from interspecific mating between those species.

## 4.1 Introduction

### 4.1.1 Spermiogenesis, chromatin remodeling, and protamines

Spermatogenesis is a highly orchestrated process that results in functional and motile sperm. The maturation of spermatids into fully functional spermatozoa occurs in the final stages of spermatogenesis, known as spermiogenesis. Here, chromatin reorganization and an increased level of compaction are essential for proper packaging of nuclear material into the sperm heads (Tokuyasu, 1972; De Kretser et al., 1998). This packaging is necessary for proper sperm head morphology, sperm motility, protection against DNA damage, and the ability to penetrate an ovum (Bianchi et al., 1993; Agarwal and Said, 2003; Aoki et al., 2005).

Unlike somatic cells, where histones serve to condense DNA, most organisms use protamines to organize DNA into a more highly condensed state within the sperm head (Ward and Coffey, 1991). Protamines increase the ability of DNA to be packed more tightly by organizing the DNA in linear, side-by-side arrays, rather than by induced supercoiling, with further stability achieved through protamine cysteine-cysteine residue interactions (Balhorn, 1982; Ward and Coffey, 1991). In mammals, transcription of protamines occur in the haploid genome, after meiosis is complete (Steger, 1999). Histones are first replaced by transition proteins TP1 and TP2, followed by protamines (Meistrich, 2003). To date, it is unclear if the haploid expression of protamines occur only in mammals, or if this allelic expression is consistent across all sexually-reproducing animals.

Extensive studies on the genes that encode for protamines have mostly been performed in vertebrates, particularly in mammalian models (reviewed in: Balhorn, 2007). With respect to invertebrates, two genes have been identified and characterized in the fruitfly, *Drosophila melanogaster*: *Mst35Ba* and *Mst35Bb*. These genes encode for *Drosophila* protamine A (protA) and protamine B (protB), respectively (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). Interestingly, *in situ* hybridization in *D. melanogaster* uncovered the presence of these protamine transcripts in primary spermatocytes (diploid cells), which have yet to undergo meiosis (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). This raises the interesting possibility that insects may differ in temporal expression of protamine genes from mammals. Furthermore, this has implications for the parental influence of protamines and their evolution: in haploid cells, only one parent contributes the genes coding for the protamines used to package the sperm head, while in diploid cells, both parental genomes may be used when transcribing protamines.

Temporal expression of protamines between two different species may also play a role in the unfit production of hybrid males. Interspecific crossing of two species often produces heterogametic (XY or ZW) hybrids that are sterile (Haldane, 1922). In hybrid males, this sterility may be due to dysfunctional protamine expression during spermatogenesis. If both species express protamine within the same spermatogenic stage, then sterile male hybrids should produce protamines in an identical temporal manner, and thus protamine

expression is unlikely to be the factor causing sterility. Alternatively, sperm of sterile hybrid males could be immotile due to differential temporal protamine expression. This could arise from differences in parental expression (i.e. parental species express protamines at different stages of spermatogenesis that do not synergize in hybrids) or from sterile male hybrids expressing protamines at a different stage of spermatogenesis than the parental species. Since protamines are incorporated during the final stages of spermatogenesis, this would suggest that post-meiotic errors are contributing to sterility in hybrid males.

Here, I present my findings on protamine production in two related species of *Drosophila*: *D. simulans* and *D. mauritiana*. To determine the parental contribution towards protamines, and thus whether they are contributed by one parent's genome (one allele) or both parent's genomes (two alleles), I use transgenic flies that produce a red fluorescent protein (RFP) or green fluorescent protein (GFP) attached to protB (Manier et al., 2010). The sperm heads of these transgenic flies emit a red or green fluorescent signal due to the tagged protamines. By crossing a male possessing the transgene of one fluorophore (e.g. RFP) with a female carrying the transgene of the other fluorophore (e.g. GFP) and examining the sperm fluorescence of the male offspring, henceforth referred to as a transgenic hybrid, we can elucidate when protamine gene expression occurs. During *Drosophila* male meiosis, the synaptonemal complex (protein structure involved in chromosome pairing) is absent and chromosomes do not undergo recombination (Morgan, 1914; Rasmussen, 1973), and thus the male offspring produced from these crosses cannot recombine the two separate transgenes onto a single chromosome in their sperm. Therefore, the sperm that is produced will only exhibit fluorescence due to either a GFP- or RFP-tagged protamine, but not both. If transcription occurs from a single allele, then we should observe a single fluorescent signal of either red or green. In contrast, dual expression of RFP or GFP within one sperm head provides evidence of diploid gene expression from both alleles.

## 4.2  Materials and methods

### 4.2.1  Fly lines and maintenance

All flies and crosses were maintained on standard Bloomington recipe media
(Bloomington Drosophila Stock Center, Bloomington, IN, USA) and flies were housed at
22°C on a 14h:10h light:dark cycle. Transgenic *D. simulans* and *D. mauritiana* flies with
GFP- and RFP-tagged protamines were kindly provided by Dr. John Belote. Transgenic
*D. simulans* lines possessed either a GFP-tagged protB (*genotype: w+; pBac{3xP3-
EGFP, ProtB-EGFP}11B*) or a RFP-tagged protB transgene (genotype*: w ; P{w8, ProtB-
DsRed-monomer, w+}3A*). Likewise, *D. mauritiana* transgenic lines also possessed either
a GFP-tagged protB transgene (genotype: w; *P{w8, ProtB-EGFP, w+}8A*) or a RFP-
tagged protB transgene (genotype: w ; *P{w8, ProtB-DsRed-monomer, w+}13A*).
Interspecies crosses were created by crossing virgin female transgenic *D. simulans*
(carrying the RFP-tagged protB transgene) with virgin male transgenic *D. mauritiana*
(carrying the GFP-tagged protB transgene).

### 4.2.2  Crosses and imaging

Five day old virgin *D. simulans* males carrying the *protB-GFP* transgene were mated
with five day old virgin *D. simulans* females carrying the *protB-RFP* transgene. The
reciprocal cross was also made. The same set of crosses was performed with equivalent
*D. mauritiana* GFP and RFP transgenic flies. Testes of newly eclosed transgenic hybrid
males (1-2 days old) were dissected in Testes Buffer (183m*M* KCl, 47m*M* NaCl, 10m*M*
Tris-HCl) and squashed using a coverslip. Images of fluorescent sperm were captured
using fluorescent imaging on a Leica DMI6000 B inverted microscope and were analyzed
using MetaMorph. Some samples were captured using Z-stacking and deconvolved with
AutoQuant deconvolution software.

I did note that transgenic flies possessing RFP-tagged protamines exhibited a lower
fluorescent intensity than those expressing GFP-tagged protamines. Therefore, contrast
and brightness levels were adjusted for some images to allow for clear visualization of the
presence or absence of fluorescence. Images of sperm with only GFP- or RFP-tagged

protamines were not adjusted; however, contrast and brightness levels of sperm from transgenic hybrids required minor changes to offer better simultaneous visualization of both fluorescent protamines.

## 4.3    Results and discussion

My results provide concrete evidence that the protamines present in sperm heads are transcribed during the diploid phase of sperm development from both alleles in the genome. This increases the likelihood that the allelic, and thus possibly overall timing, of protamine expression may vary widely across different species. Although the diploid expression of protamines could cause temporal discordance of protamine expression in hybrids, leading to the dominance of one species' protamine over the other, I find that hybrid males maintain diploid expression of protamines. The expression of protamines from two different species within a hybrid individual could allow for negative interactions and may lead to dysfunctional sperm.

Previous work on mammals found that protamines, used for packaging DNA into sperm heads, are expressed from the haploid genome after meiosis. Although it has been shown that protamines are also expressed in the insect *D. melanogaster*, and are expressed in diploid cells prior to meiosis (Jayaramaiah-Raja and Renkawitz-Pohl, 2005), it has not been shown whether this expression occurs from a single allele or if both alleles are expressed. Additionally, diploid expression has yet to be confirmed in other species of *Drosophila*. Here, I demonstrate that bi-allelic expression is seen in *D. simulans, D. mauritiana,* and their interspecies hybrids.

To ensure the dual fluorescence from RFP and GFP in the transgenic hybrids is not a product of autofluorescence, male flies with only one transgene were dissected and sperm were scored for both red and green fluorescence (Figure 4.1A-L). Transgenic flies possessing either RFP- or GFP-tagged protamines in *D. simulans* (Figure 4.1A-F), as well as *D. mauritiana* (Figure 4.1G-L), exhibited only one signal (Figure 4.1C, F, I, L). Male transgenic hybrids possessing both the GFP and RFP transgenes had sperm that exhibited both green and red fluorescence in *D. simulans* (Figure 4.2A-F) and *D. mauritiana* (Figure 4.2G-L). It was not possible to determine at which cellular stage

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protamines are expressed, since transcription of the fluorophore-labelled protein may occur at an earlier stage than translation. I can definitively say that two fluorophores are present in each sperm head, and thus expression must occur within a diploid cell. My results strongly suggest that protamine expression occurs at the diploid phase from both alleles, rather than in the haploid phase from a single allele, as observed in mammals (Oliva, 1988; Lee et al., 1987; Domenjoud et al., 1991). Separately, sterile hybrid males produced from an interspecies cross between *D. simulans* and *D. mauritiana* also exhibited bi-allelic protamine expression (Figure 4.3). Sperm of sterile hybrid males exhibited both red (Figure 4.3A) and green (Figure 4.3B) fluorescence, and clearly overlapped in their signal location (Figure 4.3C). Protamine expression within sterile hybrids occurs during the diploid stage of spermatogenesis, similar to temporal expression in the parental species. This excludes the possibility that hybrid male sterility is a by-product of incorrect temporal expression of protamines. However, this does not rule out the possibility that there is a dysregulation of the amount of protamine expression occurring within these hybrids, nor does it preclude the possibility that the protamines are improperly utilized during DNA packaging into the sperm head. It has been previously demonstrated that sterile hybrid males whose parental species are *D. simulans* and *D. mauritiana* exhibit down regulated protamine expression (Moehring et al., 2007). A possible mechanism for sterile hybrid males may be that the protamine genes are down regulated, affecting proper packaging of DNA within the sperm heads and overall fertility.

The reorganization of chromatin into a highly compact form requires the recruitment of mRNAs that were translationally repressed until spermiogenesis (reviewed in: Renkawitz-Pohl et al., 2005), such as protamines and Tpl$^{94D}$. In *Drosophila,* chromatin reorganization occurs during the canoe stage (Rathke et al., 2007), wherein three major events occur. First, histones are replaced by the transition protein, Tpl$^{94D}$, as indicated by the accumulation of Tpl$^{94D}$ transcripts prior to the presence of protamines (Rathke et al., 2007). Second, histone modification and degradation occurs, followed by DNA breaks that facilitate its unwinding (Rathke et al., 2007). Finally, protamines replace Tpl$^{94D}$ bound to DNA, permitting the high compaction (Rathke et al., 2007). Here, arginine

residues of both protamine proteins bind to chromatin, while cysteine residues link neighbouring protamine together (reviewed in: Kanippayoor et al., 2013).

Since protamines in sterile hybrid males exhibit bi-allelic expression as the parental species, *D. simulans* and *D. mauritiana* (Figure 4.3)*,* possible incompatible interactions may occur to disrupt proper packaging of sperm nuclei. Protamines derived from two different parental species within a hybrid may not interact, and as a by-product, protamines may not bind to DNA or link to neighbouring protamines. This possibility, however, seems unlikely, as a large-scale analysis identified homologs for both ProtA and ProtB in a variety of species in *Drosophila,* including two species within the *simulans* complex, *D. simulans* and *Drosophila sechellia* (Alvi et al., 2013)*.* Here, regions involved in DNA-binding were found to be highly conserved and the amino acid composition (specifically for cysteine and arginine) for both protamines was identical between *D. simulans* and *D. sechellia* (Alvi et al., 2013). Given that *D. mauritiana* is found within the same complex and the high conserved nature of protamine within *Drosophila*, it's unlikely that *D. mauritiana* harbours highly diverged protamines that would result in improper interaction with *D. simulans* protamines.

Another possible explanation for mis-packaging of sperm nuclei in sterile hybrid males, that the transition protein, Tpl$^{94D}$, may also play a factor in improper packaging of sperm nuclei. How conserved Tpl$^{94D}$ is among different species within (or outside) the genus *Drosophila* has yet to be determined. If Tpl$^{94D}$ does not degrade prior to presence of protamines, a successful transition from Tpl$^{94D}$ bound DNA to protamine bound DNA could occur, reducing the level of compaction of sperm nuclei. This could explain the uneven distribution of protamines visually observed and the decondensed sperm nuclei of sterile hybrid males (Figure 4.3). This observation, however, may also be a by-product of the mis-expression of protamines in sterile hybrid males of *D. simulans* and *D. mauritiana* (Moehring et al., 2007), as discussed in Chapter 2.

The results from this study, in addition to previous studies (Jayaramaiah-Raja and Renkawitz-Pohl, 2005; Oliva, 1988; Lee et al., 1987; Domenjoud et al., 1991), raise some interesting questions: Are there benefits between haploid *vs.* diploid expression of

protamines? Why is there a temporal difference in protamine expression between *Drosophila* and other organisms? Perhaps the answer lies in the sharing of haploid-expressed transcripts between connected sperm heads. In mammals, protamine transcripts are shared through cytoplasmic bridges connecting the non-individualized sperm after meiosis is complete (Caldwell and Handel, 1991). Even though each protamine is only transcribed from the haploid genome, the individual sperm has access to the transcripts from the diploid genome due to these cytoplasmic bridges. It is possible that non-individualized sperm heads are not equally sharing postmeiotic transcripts, so it is unclear what the degree of access to both protamines truly is within each sperm head (Lee et al., 1995). If sharing is unequal, subtle differences in sperm head packaging may exist between individualized sperm heads due to differences in the protamine allele that is present in each sperm's haploid genome. This could have a profound effect on the sperm's fertilization success and the individual's overall fitness (Immler, 2008), resulting in strong purifying selection on protamine alleles. In contrast, organisms with protamine expression prior to meiosis from the diploid genome will ensure equal protamine transcripts across all sperm heads, and thus individual protamine alleles may have a lesser impact on sperm function. This would prove to be especially important for species that are polygamous and undergo sperm competition within the reproductive tract (Parker, 1970; Snook, 2005).

The expression of protamines during either the haploid or diploid phase in different species may indicate that there are benefits or costs to expression during one phase compared to the other. There may be ramifications of haploid gene expression that are alleviated by diploid expression. For example, protamine expression during the haploid phase may cause sperm from a single male to be more phenotypically different from each other, as well as from the diploid male (Immler, 2008). As such, sperm derived from one male may potentially compete with each other, setting up a conflict of interest between the sperm and the male, as each sperm competes to successfully fertilize the egg, potentially affecting the male's ability to maximize his own fitness (Parker, 1993; Parker and Begon, 1993). Further studies may identify an advantage of protamine expression in the haploid *vs*. diploid phase, and how species benefit uniquely to one expression pattern over the other.

Although many stages within spermiogenesis are conserved between *Drosophila* and mammals, there are major differences, including the findings from this paper, on the timing and genomic contribution towards protamine expression. Mice and humans have two protamines that likely arose due to a gene duplication event (Domenjoud et al., 1990; Hecht et al., 1986).These genes require two fully functional copies in order to prevent male sterility (Cho et al., 2001). *Drosophila* also possesses two protamine genes but each copy is not haploinsufficient (Rathke et al., 2010). In determining the functional significance of the protA and protB genes, it was surprising to discover that male flies with homozygous deletions for both protamine genes at the same time did not have a reduction in sperm motility or fertility, although approximately 20% had abnormally-shaped nuclei, suggesting some level of protamine functional redundancy (Rathke et al., 2010). Although fertility was not greatly impacted in these mutant flies, sperm that lacked both protA and protB were more sensitive to X-ray mutagenesis, indicating that the protamines may serve to protect DNA from damage in *Drosophila* (Rathke et al., 2010).

Aside from the implications that sperm packaging has for male fertility, understanding DNA condensation and proper sperm head packaging also has applications from an evolutionary perspective, since there will be different selective pressures on a gene that is expressed only in a haploid state from those that are expressed in a diploid state (Jenkins, 1993; Joseph and Kirkpatrick, 2004). To understand the extent of differential protein expression in sperm heads, additional work in characterizing protamines across different taxa will need to be completed to further understand the evolutionary implications of diploid *vs.* haploid gene expression. For example, we can use an anti-GFP nanobody to target and knock out protamines that fused to GFP (Caussinus et al., 2011) and reduce the levels of expression of protamines bound to GFP in our *Drosophila* lines. In this instance, we can study the effects of haploid protamine expression in a biological system that normally exhibits diploid protamine expression.

**GFP** **RFP** OVERLAY
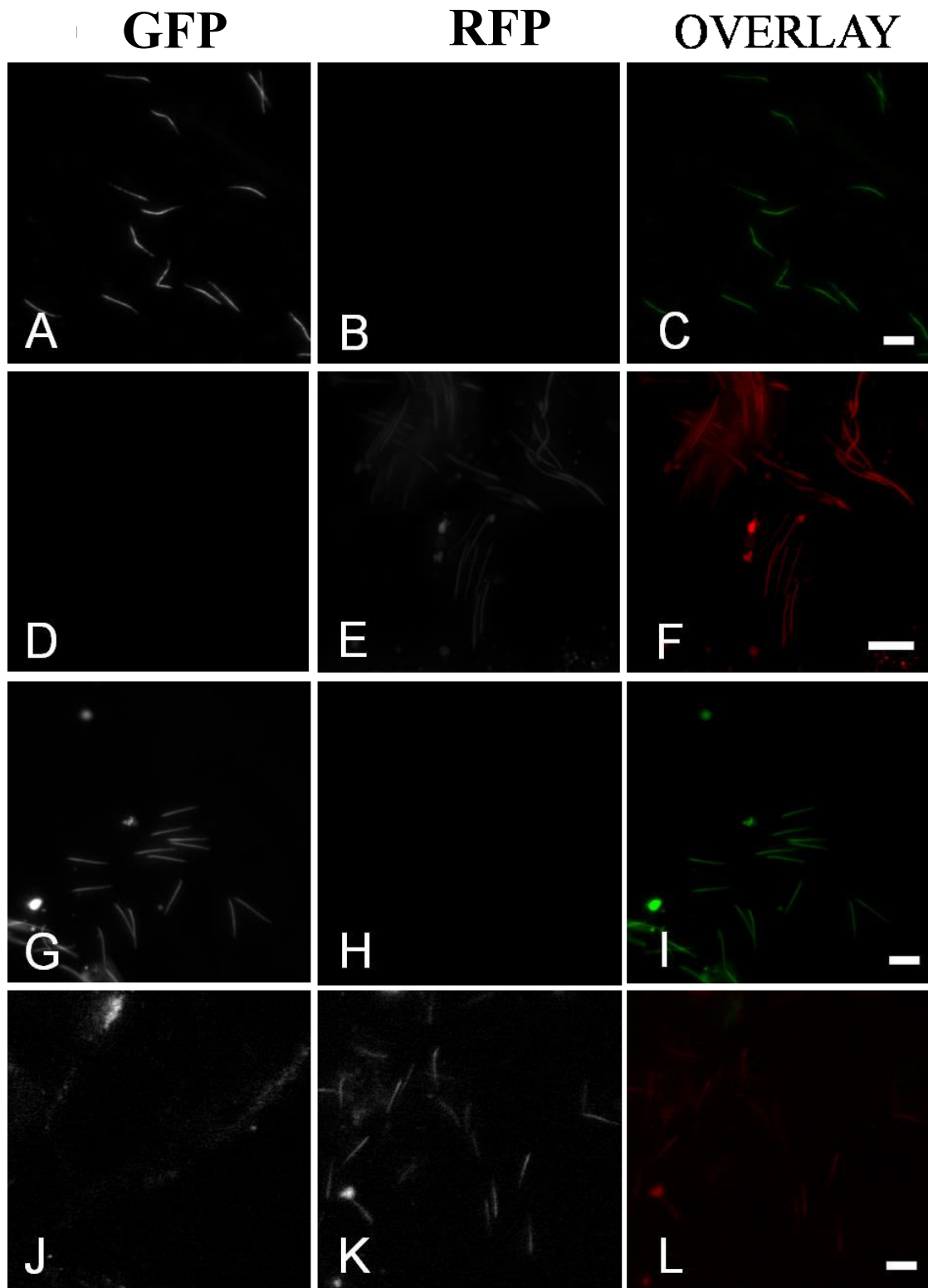
Figure 4.1: *D. simulans* and *D. mauritiana* exhibit bi-allelic protamine expression. *D. simulans* (A-F) and *D. mauritiana* (G-L) sperm heads possessing GFP-tagged (A-C, G-I) or RFP-tagged (D-F, J-L) protamine. Sperm containing the protB-GFP transgene only fluoresce green (A, C, G, I) and do not reveal any red light autofluorescence (B, C, H, I). Similarly, sperm containing only the protB-RFP transgene only fluoresce red (E, F, K, L), with no green auto-fluorescence (D, F, J, L). Images A-C and G-L were taken at 40x magnification, while images D-F were taken at 63x magnification. Bars represent 10µm.
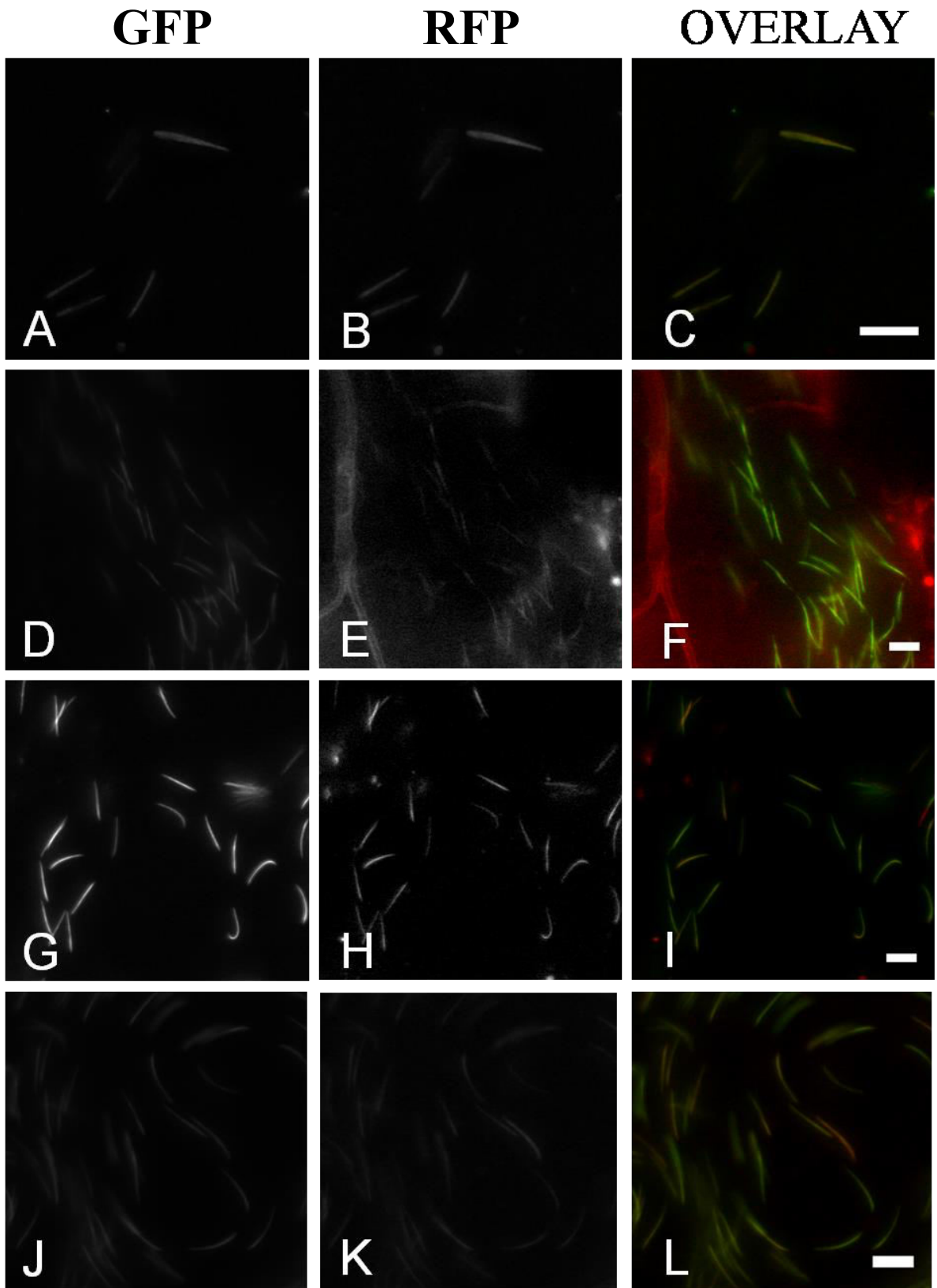
Figure 4.2: *D. simulans* and *D. mauritiana* exhibit bi-allelic protamine expression. Transgenic hybrids in *D. simulans* (A-F) and *D. mauritiana* (G-L). *D. simulans* females with the transgene possessing the GFP-tagged protamine mated to *D. simulans* males with RFP-tagged protamine transgene (A-C) and the reciprocal cross (D-F) fluoresce both red and green (C, F). Similarly, *D. mauritiana* females with the transgene possessing the GFP-tagged protamine mated to *D. mauritiana* males with RFP-tagged protamine transgene (G-I) and the reciprocal cross (J-L) also fluoresce both red and green (I, L), thus suggesting that protamine expression occurs during the sperm cell's diploid phase. Images A-C were taken at 63x magnification, while images D-L were taken at 40x magnification. Bars represent 10μm.

GFP RFP OVERLAY
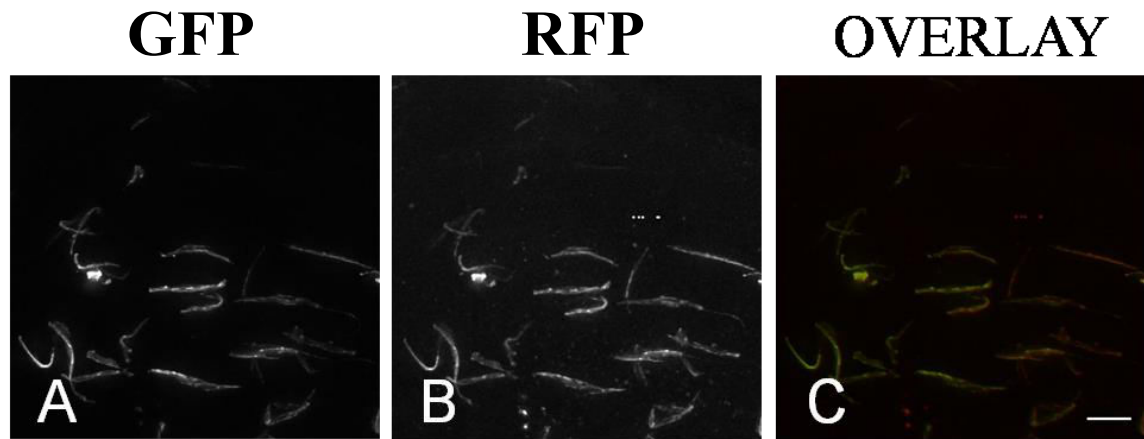
Figure 4.3: Sterile hybrid males produced from *D. simulans* mated to *D. mauritiana* exhibit bi-allelic protamine expression. Sterile hybrids from interspecific cross between *D. simulans* and *D. mauritiana*. Female *D. simulans* with the RFP-tagged protamine transgene were mated to male *D. mauritiana* with the GFP-tagged protamine transgene. Sterile hybrid males produce sperm exhibiting both red (A) and green (B) fluorescence. Fluorescent signals from both RFP and GFP transgenes overlap in location (C), suggesting similar temporal protamine expression as the parental species. Images A-C were taken at 63x magnification. Bar represents 10μm.

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# Chapter 5

# 5 Identification of a novel sperm phenotype in sterile male hybrids in *Drosophila*

The genetic architecture of hybrid sterility has been extensively studied, especially in *Drosophila.* The two prevailing theories on the evolution of $F_1$ hybrid sterility and its link to Haldane's rule (HR) remain controversial. Genetic mapping has uncovered genetic regions implicated in hybrid sterility, but neither individual candidate genes nor a common genetic mechanism have been identified. Here, I identified a novel phenotype that I call needle-eye sperm, and demonstrate that it is present in sterile hybrid males of at least three separate species pairs of *Drosophila*. This phenotype was then genetically mapped by a series of backcrosses (BC) to either *D. simulans* or *D. mauritiana* for 10 generations. Using next-generation sequencing (NGS) and *p*henotype-based *s*election and *i*ntrogression followed by whole-genome re*s*equencing (PSIseq), no significant regions have been linked to the formation of needle-eye sperm. However, small regions that contained introgressed *D. simulans* or *D. mauritiana* were found to be unique in sterile hybrid males for each backcross. Due to this potentially common phenotype among sterile male hybrids suggested by sperm morphology*,* further exploration of the genes uncovered here may identify a universal mechanism for the evolution of hybrid sterility.

## 5.1  Introduction

### 5.1.1  Hybrid sterility and Haldane's rule

The Bateson-Dobzhansky-Muller incompatibility model, herein referred to as the BDM model, has been the predominant model used to explain the evolution of postzygotic reproductive isolating barriers (Bateson, 1909; Dobzhansky, 1936; Muller, 1940). Allopatric populations that evolve independently of each other, acquire different mutations (Dobzhansky, 1936; Muller, 1940). Genetic differences between each population can become fixed, as mutations are subjected to natural selection and genetic drift (Lynch and Force, 2000). For a given population, the genetic differences may appear as karyotypic, coding, or non-coding changes (Maheshwari and Barbash, 2011), ultimately altering each population's genetic makeup. While these genomic changes are

compatible within each population, they may be incompatible in the genetic background of the other population, which has evolved on an independent trajectory (Bateson, 1909; Dobzhansky, 1936; Muller, 1940). Therefore, if hybrid offspring are produced from a mating event between genetically incompatible populations, those hybrids can have reduced fitness due to these genetic incompatibilities (e.g. Coyne and Orr, 1989; Fishman and Willis, 2000; Mihola et al., 2009). If these reproductively isolating barriers have evolved between the two populations, gene flow will be reduced or eliminated, and over time these populations will continue to evolve and become distinct species (Presgraves 2010).

An individual produced from the hybridization of two divergent, and genetically incompatible, populations may exhibit developmental defects that render the individual sterile. Hybrid sterility, an intrinsic postzygotic reproductive barrier, has been noted in many interspecific crosses (e.g., Coyne and Orr, 1989; Presgraves and Orr, 1998; Fishman and Willis, 2000; Coyne and Orr, 2004; Mihola et al., 2009; Presgraves 2010). A notable trend observed across multiple interspecies pairs, is the asymmetrical prevalence of sterility between the hybrid sexes. If only one sex is affected by postzygotic isolation, heterogametic (e.g., XY or ZW) individuals are more likely to be sterile than homogametic (XX or ZZ) individuals. This trend is referred to as Haldane's rule (HR; Haldane, 1922). Since HR is seen among almost all interspecies hybrids, a common genetic basis for the evolution of hybrid sterility may exist across multiple taxa.

## 5.1.2 The genetic basis of hybrid sterility and Haldane's rule: the Dominance theory

There are a number of theories that have been developed to explain the presence of Haldane's rule and hybrid sterility, wherein the most recognized theories are: Dominance theory, Faster male, Faster X, and meiotic drive (Coyne and Orr 2004). Researchers have provided some empirical support for each theory (Perez et al., 1993; Wu et al., 1996; Presgraves and Orr, 1998; Henikoff et al., 2001; Coyne and Orr, 2004; Sun et al., 2004; Phadnis and Orr, 2009; Phadnis, 2011), with no single theory emerging as a universal explanation for Haldane's rule and sterility. However, among these theories, the Dominance theory is most common (Presgraves, 2010). The Dominance theory describes

that physically separate populations acquire different mutations that are compatible within their respective genetic backgrounds (Maheshwari and Barbash, 2011). Upon hybridization, an incompatible interaction between a recessive sex-linked locus of one species, with a dominant autosomal-linked locus of another species results in developmental defects of a hybrid, rendering the hybrid sterile (Orr and Turelli, 1996). As the sex-linked locus is recessive, then heterogametic hybrids are disproportionately affected over homogametic hybrids, due to the presence of a single copy of each sex chromosome in heterogametic hybrids (Orr and Turelli, 1996).

Identifying the loci responsible for HR is difficult in older species, as genetic incompatibilities continue to accumulate over the course of speciation (Matute et al., 2010). However, much research has gone into uncovering the loci (or genes) that give rise to HR. In *Drosophila,* the most extensively used genus in studies of species isolation, genes for hybrid inviability provide strong support for the Dominance model as a common mechanism underlying hybrid dysfunction (Bateson, 1909; Dobzhansky, 1936; Muller, 1940; Orr and Turelli, 1995). In contrast, none of the genes identified for hybrid sterility in *Drosophila* fully satisfy the Dominance theory, as a recessive-dominant two-locus incompatibility (Perez et al., 1993; Perez and Wu, 1995; Masly et al., 2006), with the potential exception of the gene *Overdrive* (Phadnis and Orr, 2009; Phadnis, 2011).

## 5.1.3    Overview of study

If the Dominance theory is correct, random genetic changes that accumulate within a population would lead to sterility when introduced into the genetic background of a different population. In *Drosophila*, we would expect males to be more susceptible to hybrid sterility than females if these random genetic incompatibilities were more commonly found on the X chromosome (Mallet et al., 2011; Civetta, 2016), or if spermatogenesis is more sensitive to genetic disruptions than other processes involved in development (Wu et al., 1996). In both of these scenarios, however, genetic mutations that lead to hybrid sterility in different species pairs are unlikely to affect the same point in spermatogenesis as in the sterile hybrid produced by each pair.

My previous work refining the phenotypic characteristics of sterile hybrid males in *Drosophila* found a trend among three interspecies crosses (see Chapters 2 and 3). Specifically, these studies identify that errors during spermatogenesis arise during meiosis, yet the overall process of spermatogenesis continues within these hybrid males (Kulathinal and Singh, 1998), resulting in half the number of sperm produced (Chapter 2), yet these sperm appear alive with functional mitochondria (Chapter 3). Further inspection of individual sperm at the ultrastructural level suggests that a single sperm may be harbouring two sperm tails (Chapter 2), or sperm are paired and unable to separate.

Here, I identify a novel sperm phenotype in sterile hybrid males within three interspecies crosses in *Drosophila,* suggesting a potential universal phenotype. The nuclei of sperm produced by a sterile hybrid male appear paired, thus I have coined this previously unidentified phenotype as "needle-eye sperm". To identify the genomic regions that are responsible for the formation of needle-eye sperm, phenotype based selection was performed on sterile hybrid males produced from backcrossing (BC) for 10 generations to either *Drosophila simulans* (BCS10) or *Drosophila mauritiana* (BCM10). Males from each backcross type were pooled into one of two phenotypes: produce needle-eye sperm, does not produce needle-eye sperm. All four pooled samples were then sequenced using Illumina technology and raw sequences were analyzed using *p*henotype-based *s*election and *i*ntrogression followed by whole-genome re*seq*uencing (PSIseq) to identify introgression breakpoints (Earley and Jones, 2011). By locating the introgression boundaries using PSIseq, I can determine the presence of *D. simulans* within the genetic background of *D. mauritiana* and vice versa. Three autosomal regions on the 3rd and 2nd chromosomes were identified as being associated with the formation of needle-eye sperm, with one region (2L) found in both backcrosses of *D. simulans* and *D. mauritiana.*

## 5.2      Materials and methods

### 5.2.1     *Drosophila* lines/husbandry and interspecies crosses

All *Drosophila* lines and interspecies crosses were maintained on standard Bloomington media (Bloomington Drosophila Stock Center, Bloomington, IN, USA) and were housed

at 22$^0$C on a 14h:10h light:dark cycle at 75% humidity. *Drosophila mojavensis* (#1501-1352.22)*, Drosophila arizonae* (#15081-1271.00)*, Drosophila pseudoobscura* (#114011-0121.149)*,* and *Drosophila persimilis* (#14011-0111.49) were obtained from the *Drosophila* Species Stock Center (San Diego, CA, USA). Transgenic *D. simulans* and *D. mauritiana* flies, both with GFP-tagged protamine B (genotype: *w$^+$; pBac{3xP3-EGFP, ProtB-EGFP}11B*) were obtained from Dr. John Belote. Henceforth, transgenic *D. simulans* and *D. mauritiana* with GFP-tagged protamine B will be denoted as *simGFP* and *mauGFP*, respectively.

Interspecific crosses were only performed between species whose hybrids produced sperm. Female *D. mojavensis* were mated with male *D. arizonae,* and female *D. pseudoobscura* were mated to male *D. persimilis*, as well as the reciprocal crosses of those species pairs. Female *simGFP* and male *mauGFP* were mated; the reciprocal cross was not performed as it produces hybrid males that do not produce sperm (Kulathinal and Singh, 1998).

## 5.2.2    Sperm nuclei staining and fluorescent microscopy

Sterile hybrid males produced from the interspecific cross between *D. arizonae* and *D. mojavensis* (offspring denoted as *ari/moj*) and *D. pseudoobscura/D. persimilis* (offspring denoted as *pse/per*) were collected upon eclosion and aged for one day. Testes were removed in approximately 200 µL of Testes Buffer (183m*M* KCl, 47m*M* NaCl, 10m*M* Tris-HCl) and immediately transferred to 20µL of 0.5µl/ml of 4',6-diamidino-2-phenylindole (DAPI), ripped open to allow contents to expel, and incubated for 5 mins on a siliconized cover slip. Testes contents were washed using Testes Buffer three times, then mounted on a glass slide. Testes of sterile hybrid males produced from the interspecific cross between *D. simulans GFP* and *D. mauritiana GFP* (offspring denoted as *sim/mau*) were extracted using Testes Buffer and mounted on a glass slide.

Images were taken using a Leica DMI6000 B inverted microscope and were analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Some samples were captured using Z-stacking with images de-convolved with AutoQuant software (AutoQuant Imaging, Inc).

### 5.2.3    Phenotype-based selection and introgression by backcrossing

Young (2 days old) virgin female *simGFP* were mated to same-age *mauGFP* males, and hybrid daughters were collected and aged 2 days. $F_1$ hybrid females were singly mated back (i.e., first generation backcrossed; BC1) to either one *simGFP* male (BCS1) or one *mauGFP* male (BCM1; Figure 5.1). Virgin daughters and sons that were produced were separated. Males were aged for 1 day and then scored for "needle-eye sperm" or "fertile sperm". Fertile sperm are sperm that are motile and containing a single nucleus. If a single mating pair produced some or all sons with needle-eye sperm, their daughters were used for the next generation of backcrossing (i.e. BC2). This phenotype-based selection (needle-eye sperm and fertile sperm) and introgression mapping was performed for 10 generations of backcrossing (i.e. to BC10).
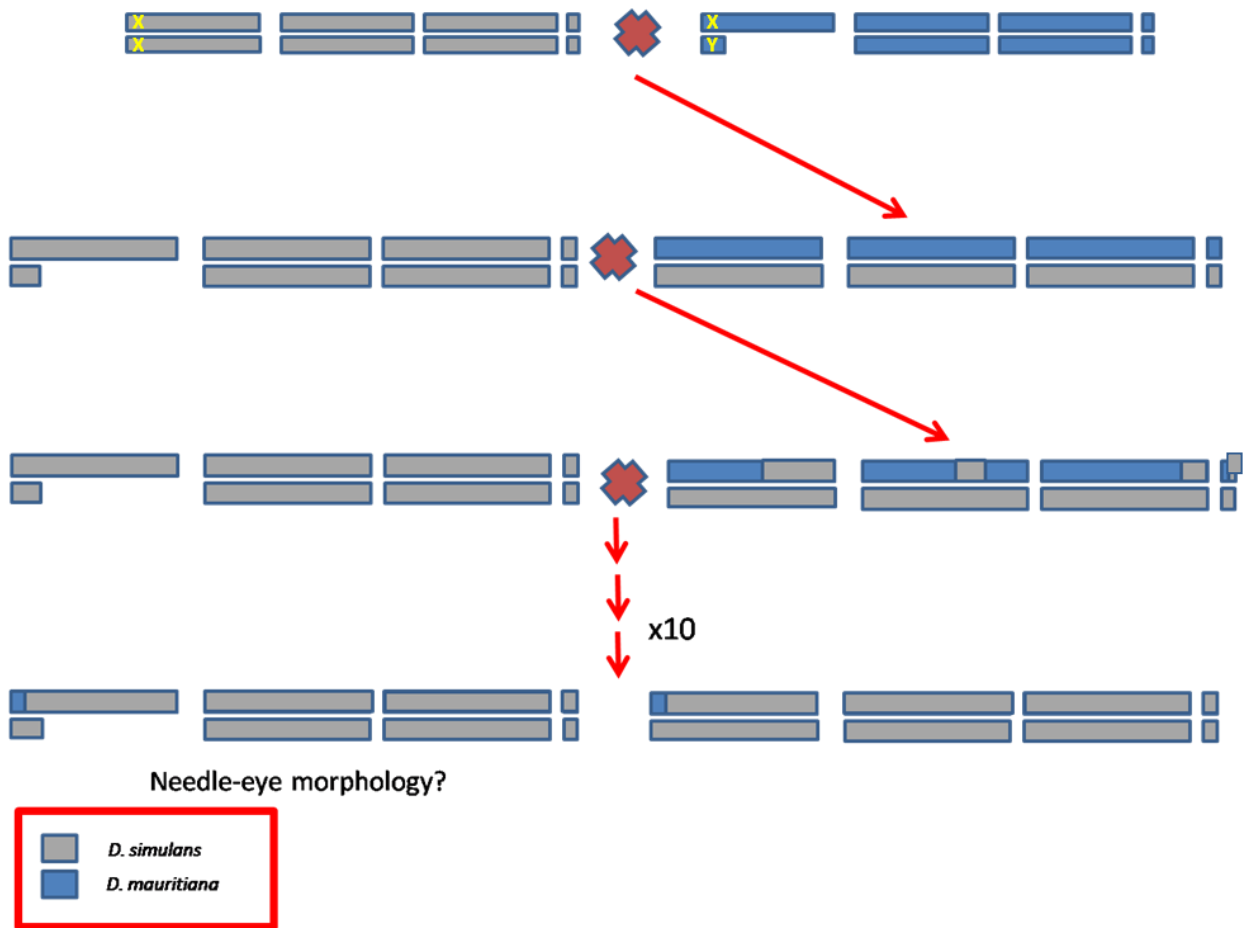
Figure 5.1: Crossing scheme for the generation of hybrid sons producing either needle-eye sperm (sterile) or motile, non-needle-eye sperm (fertile). In this figure, a single daughter, produced from a cross between *D. simulans* (female) with *D. mauritiana* (male; X chromosome is paired with a shorter chromosome, Y), was then backcrossed to a single male *D. simulans*. Sons were scored for the presence of needle-eye sperm and their sisters were used to singly mate with a male *D. simulans*. This process was repeated for 10 generations of backcrossing. This crossing scheme was repeated for the reciprocal cross (not shown), wherein an F$_1$ daughter produced from the mating between female *D. simulans* with male *D. mauritiana* was backcrossed to *D. mauritiana* for 10 generations.

## 5.2.4    Bulked segregation analysis and next-generation sequencing

Males produced after 10 generations of backcrossing to *simGFP* (BCS10) or *mauGFP* (BCM10) were scored for either needle-eye sperm or fertile sperm. Thirty BCS10 males from a single surviving lineage with needle-eye sperm (BCS10NE) were pooled together, and 30 BCS10 males from the same single lineage with fertile sperm (BCS10F) were pooled together for DNA extraction. The same procedure was done with BCM10 males (from five lineages) for both needle-eye sperm and fertile sperm (BCM10NE and BCM10F, respectively), totaling four different phenotypes with pooled individuals for each phenotype. Only 30 males were pooled for each phenotype, as more individuals would reduce the efficacy of the DNA extraction process and overall yield.

DNA extraction for each phenotype was performed as a modified protocol of the QIAGEN Gel Extraction Kit (Qiagen, Toronto, ON, Canada). Each sample was held at $95^0$C for 5 mins in a buffer solution (1M Tris-HCl, 0.5M EDTA, and 5M NaCl) containing 200µg/mL Proteinase K. Isopropanol was then added to each sample and incubated overnight at -20 $^0$C. DNA purification was performed as directed by the QIAGEN Gel Extraction Kit. Purified DNA samples were then sequenced using the Illumina HiSeq 2000 PE 100 at Génome Québec Innovation Center (Montréal, QC, Canada).

## 5.2.5    Assembly of genomic DNA for *D. simulans* GFP and *D. mauritiana* GFP

Assembly of the FASTQ files generated by Illumina sequencing technology was performed using a Linux-based *de novo* assembler, SOAP denovo. Scaffold assemblies were performed by Dr Tom Hsiang. Scaffolds were not further assembled into chromosomes.

## 5.2.6    Sequence analysis using PSIseq pipeline

FASTQ sequences of all four phenotypes (BCS10NE, BCS10F, BCM10NE, and BCM10F) were analyzed using the PSIseq pipeline to identify loci contributing to needle-eye sperm (Earley et al., 2011). PSIseq generates vertical alignment files to identify SNPs that are unique to either parental population. In this step, the genomes of *simGFP* and

*mauGFP* are aligned using BWA to create a Parent1-Parent2 SNP database. Using the raw sequence reads for the four studied phenotypes, an identical alignment via BWA was used to identify hybrid-parent mismatches. For example, the reads derived from BCS10NE were aligned to the sequences of *simGFP* to create the hybrid-parent mismatches. Upon identifying the positions of Parent1-Parent2 SNPs, a "window" approach, using 1000 SNPs/window, was used to identify the prevalence of those hybrid-parent mismatches within a given sequence length (Huang et al., 2009). Within each window, the number of hybrid-parent mismatches was determined. The likelihood that a given stretch of sequences belongs to Parent 1 *versus* Parent 2 is determined by a binominal distribution (Earley and Jones, 2011). This approached uses multiple independent significance tests and as such, was corrected using a false discovery rate (Earley and Jones, 2011). This method efficiently identifies introgression regions of the parent's genome that have not been lost due to the recombination backcrossing method.

A follow-up study was performed to identify the top 10 regions that contained *D. simulans* SNPs within a genetic background of *D. mauritiana* that was uniquely found in males with needle-eye sperm (sterile) *versus* no needle-eye sperm (fertile). A reciprocal study was performed wherein regions containing *D. mauritiana* SNPs were identified within the genetic background of *D. simulans* in males with needle-eye *versus* no needle-eye sperm. Using PSIseq, I extracted the file that described whether a given SNP in a scaffold was heterozygous for *D. simulans* and *D. mauritiana* (hybrid SNP) or homozygous for either backcross parent (e.g. *D. simulans* in BCS10). Regions with hybrid SNPs that were found to occur only in needle-eye males, but not fertile males, were kept and ranked based on the number of hybrids SNPs found in each scaffold. The 10 scaffolds with the most SNPs were further analyzed to identify chromosomal location and the presence of any genes.

## 5.3    Results

### 5.3.1    Sperm head morphology and "needle-eye" sperm

Sperm head morphology was examined in young (<2 days old) males for six different species of *Drosophila* and their respective interspecies hybrids (Figure 5.2). As expected,

all sperm heads of each pure species (N=10) possessed properly separated nuclei (Figure 5.2A, 5.2B, 5.2D, 5.2E, 5.2G, 5.2H). Ten sterile hybrid males produced between *D. simulans* and *D. mauritiana* possess sperm with heads that appear attached at oppose ends of the nuclei, with a void between both heads (Figure 5.2C). As this is the first documentation of this sperm morphology in interspecific sterile hybrid males, I have denoted this phenotype as "needle-eye" sperm, as it resembles the eye of a sewing needle. The production of needle-eye sperm is also apparent for all other interspecific crosses between *D. pseudoobscura* and *D. persimilis* (Figure 5.2F), as well as hybrids produced from *D. arizonae* and *D. mojavensis* (Figure 5.2I). Interestingly, sperm from hybrids of the *pse/per* cross did not exhibit two different sperm lengths (i.e. parasperm and eusperm); rather, sperm appeared similar, contrary to what is observed in pure species. Sperm from sterile male hybrids were taken at 63x magnification instead of 40x magnification to ensure proper visualization of two-headed sperm. Sperm from pure species males were, however, also observed at 63x magnification to ensure the absence of needle-eye sperm (data not shown).
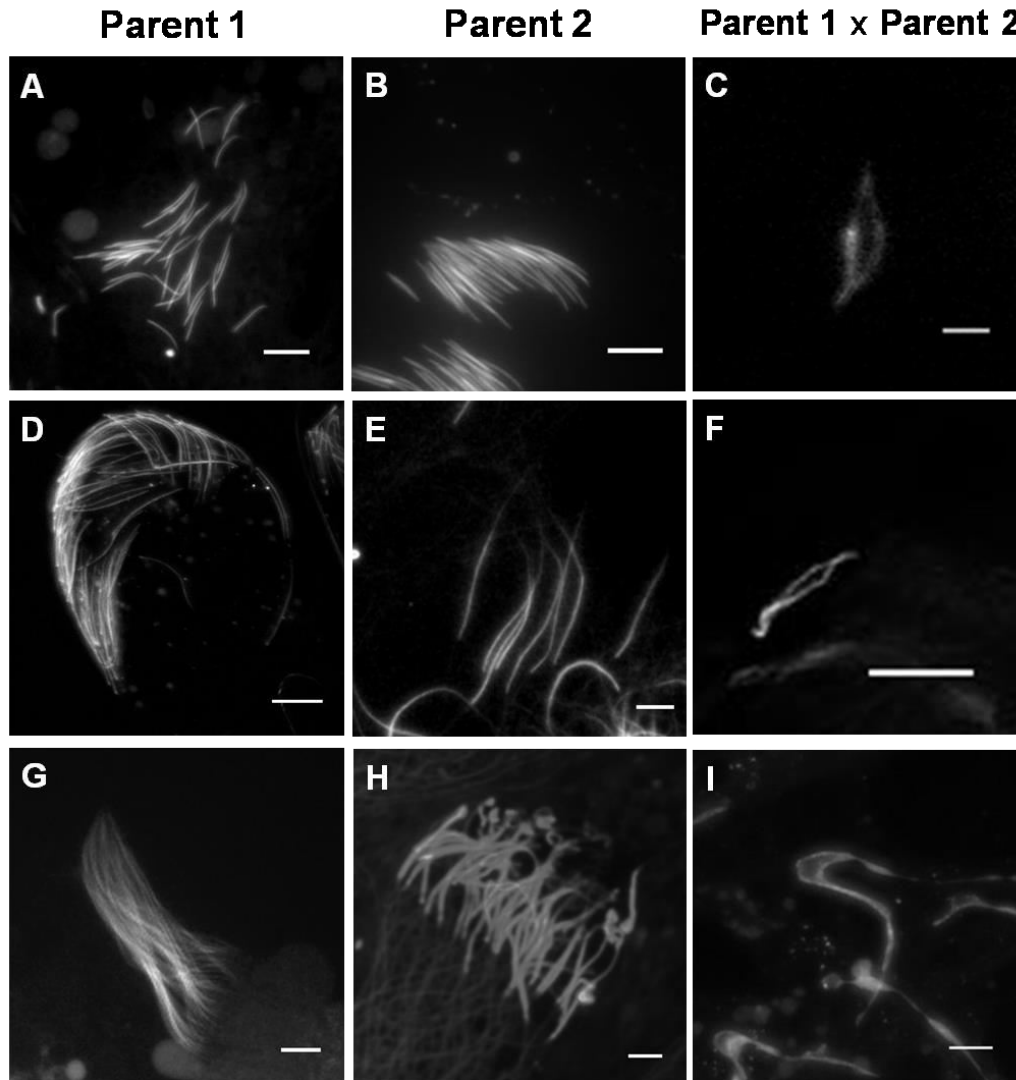
Figure 5.2: Novel sperm phenotype in sterile hybrid males in all interspecies crosses in *Drosophila.* Fluorescent microscopy of sperm cells in 6 species of *Drosophila* and their interspecific sterile hybrid males: *D. simulans* (A), *D. mauritiana* (B), *sim/mau* (C), *D. pseudoobscura* eusperm (D), *D. persimilis* eusperm (E), *pse/per* (F), *D. arizonae* (G), *D. mojavensis* (H), *ari/moj* (I). Sperm nuclei of males from each parental species are separate within a sperm bundle or ejaculate, whereas sperm nuclei of sterile hybrid males appear paired at polar ends of the nuclei, denoted as needle-eye sperm. Panels C, F, I were taken at 63x magnification, while all other panels were taken at 40x magnification. Scale bars for C, F, and I represent 0.05mm, while all other scale bars represent 0.01mm.

## 5.3.2 Prevalence of needle-eye sperm in BCS10 and BCM10 males

After 10 generations of backcrossing to either *D. simulans* or *D. mauritiana,* the number of sons producing needle-eye sperm for each mother was scored (Table 5.1). The four females that were singly mated to a male *D. simulans* that produced some sterile sons produced roughly 50% BCS10 sons with needle-eye sperm and 50% with wild-type sperm (Table 5.1). All other females produced sons with only fertile sperm (data not shown). Similarly, the 15 females that were singly mated to a male *D. mauritiana* and produced some sterile sons produced roughly 50% BCM10 sons with needle-eye sperm and 50% with wild-type sperm (Table 5.1). The difference in the number of females mated between BCM and BCS is due to the total pool of females available for crossing. All other females produced sons with only fertile sperm (data not shown). Thus, after 10 generations of backcrossing, there was a Mendelian pattern of inheritance, indicating that a single region may be responsible for the formation of needle-eye sperm in either BCM or BCS sterile males.

Table 5.1. Prevalence of needle-eye sperm in BCS10 and BCM10 males. Females with brothers who produced needle-eye sperm were singly mated to either a male *D. simulans* or a male *D. mauritiana*, creating BCS10 or BCM10 sons, respectively. If a female produced BCS10 sons with needle-eye sperm, roughly half of her progeny exhibited needle-eye sperm. A similar effect was observed

| Backcross | Female | Number of sons scored | Number of sons with needle-eye sperm | Percentage of sons produced with needle-eye sperm |
|---|---|---|---|---|
| BCS10 | 1 | 9 | 4 | 44 |
| | 2 | 30 | 15 | 50 |
| | 3 | 9 | 5 | 56 |
| | 4 | 20 | 10 | 50 |
| BCM10 | 1 | 10 | 5 | 50 |
| | 2 | 7 | 4 | 57 |
| | 3 | 8 | 4 | 50 |
| | 4 | 6 | 3 | 50 |
| | 5 | 10 | 5 | 50 |
| | 6 | 10 | 5 | 50 |
| | 7 | 10 | 5 | 50 |
| | 8 | 10 | 4 | 40 |
| | 9 | 10 | 4 | 40 |
| | 10 | 10 | 4 | 40 |
| | 11 | 10 | 5 | 50 |
| | 12 | 10 | 4 | 40 |
| | 13 | 10 | 5 | 50 |
| | 14 | 10 | 5 | 50 |
| | 15 | 6 | 5 | 83 |

in females who produced BCM10 sons.

### 5.3.3 PSIseq and introgression regions found in BCS and BCM

No identifiable scaffolds were significantly enriched with *D. mauritiana* or *D. simulans* SNPs. As such, no regions along the *D. simulans* genome significantly contained introgressed regions of *D. mauritiana* and vice versa.

Using PSIseq, I did identify scaffolds that contained SNPs differentiating the two species. The ten scaffolds that contained the largest number of *D. mauritiana* SNPs in BCS males with needle-eye sperm was isolated. Each scaffold sequence was then analyzed using BLAST homology searches to identify the chromosomal location of the scaffold and any candidate genes it may harbour (Table 5.2). Similarly, I used PSIseq to determine ten scaffolds that contained the largest number of *D. simulans* SNPs only in needle-eye males of BCM. These ten scaffolds were also analyzed using BLAST to identify any possible genes and chromosomal location (Table 5.2).

The top ten scaffolds of sterile hybrid males produced after ten generations of backcrossing to *D. simulans* possess regions of *D. mauritiana* SNPs within the second and third chromosomes. Candidate genes on the second chromosome include *GD25754, GD23830, GD10073, GD10104, GD10071.* Candidate genes found within the third chromosome are *GD13646, GD18265, GD20661, GD29090, GD20533.* Four genes are of unknown function; the remaining six candidate genes have diverse functions, and no functional trends are apparent.

Sterile hybrid males produced from ten generations of backcrossing to *D. mauritiana* possess regions of *D. simulans* SNPs within the X, second and third chromosomes. One region was identified on X chromosome based on sequence similarity to *D. melanogaster* and contained the gene *Notch.* Five candidate genes found within the second chromosome were *Heterochromatin protein 6* (*HP6*)*, Thioester-containing protein 2* (*TEP2*)*, monkey king protein* (*mkg-p*)*, GD25593*, and *Amyrel.* The third chromosome also contains four candidate genes that were identified based on sequence similarity to *D. melanogaster: Integrator 11* (*IntS11*)*, alphabet* (*alph*)*, TATA-box binding protein associated factor 1* (*TAF1*), and *Sensory neuron membrane protein 2* (*snmp2*).

Table 5.2.Genes within the regions of introgression for 10 generations of backcrossing to either *D. mauritiana* (BCM10) or *D. simulans* (BCS10).

| Backcross | Scaffold[1] | Scaffold size (bp) | Chromo. | Candidate Gene[2] | Molecular function[3] | Biological Process[3] |
|---|---|---|---|---|---|---|
| *Drosophila simulans* | scaffold1 | 591 | 2L | *Umbrea* (*HP6*) | chromatin binding | female meiotic division, telomeric capping |
| | scaffold2 | 1713 | 2L | *Thioester-containing protein 2* | endopeptidase inhibitor activity | phagocytosis |
| | scaffold3 | 805 | 2L | *monkeyking protein* | nucleotidyltransferase activity | unknown |
| | scaffold4 | 215 | 3R | *TATA-box binding protein associated factor 1* | histone acetyltransferase activity | histone acetylation (H3-K14 and H4) |
| | scaffold5 | 559 | 3R | *Integrator 11* | neurogenesis | mRNA processing (cleavage, polyadenylation) |
| | scaffold6 | 516 | 3R | *alphabet* | phosphatase activity | Mg ion binding |
| | scaffold7 | 818 | 2R | *GD25593* (*Gpo-1*) | Calcium ion binding, G3P dehydrogenase activity | G3P metabolic process |
| | scaffold8 | 217 | 2R | *Amyrel* | catalytic activity, cation binding | carbohydrate metabolic process |
| | scaffold9 | 508 | 3L | *Sensory neuron membrane protein 2* | unknown | sensory perception of pain |
| | scaffold10 | 761 | X | *Notch* | chromatin binding | receptor activity |
| *Drosophila mauritiana* | scaffold1 | 648 | 2R | *GD25754*(*Vmat*) | drug transmembrane transporter activity | drug transport |
| | scaffold2 | 569 | 3L | *GD13646*(*mRpL46*) | hydrolase activity | unknown |
| | scaffold3 | 526 | 2L | *GD23830*(*CG31862*) | unknown | unknown |
| | scaffold4 | 431 | 2R | *GD10073*(*ced-6*) | unknown | unknown |

| | | | | | |
|---|---|---|---|---|---|
| scaffold5 | 684 | 3R | *GD18265(RabX4)* | GTP binding | small GTPase mediated signal transduction |
| scaffold6 | 618 | 2R | *GD10104(Pmm45A)* | transferase activity, phosphotransferases | carbohydrate metabolic process |
| scaffold7 | 493 | 3R | *GD20661* | transporter activity | transport |
| scaffold8 | 228 | 3R | *GD20533(grsm)* | aminopeptidase activity, Mn ion binding | proteolysis |
| scaffold9 | 234 | 3R | *GD29090* | unknown | unknown |
| scaffold10 | 334 | 2R | *GD10071(CG1888)* | unknown | unknown |

[1] Scaffolds are numbered from those having the more introgressed heterospecific SNPs (1) to those having fewer SNPs (10).

[2] Candidate gene names are listed with the *D. melanogaster* homolog in parentheses; if no parentheses are listed then the gene name is identical to the name in *D. melanogaster*.

[3] Molecular functions and biological processes are for the *D. melanogaster* homolog; this information is not known for *D. simulans* and *D. mauritiana*.

## 5.4 Discussion

Decades of research have been invested in uncovering the genetic basis of hybrid sterility, specifically in the genus *Drosophila*, yet few genes have been uncovered and none support a universal mechanism (Presgraves, 2010). Here, I have uncovered a previously uncharacterized sperm phenotype for sterile hybrid males across multiple interspecies pairs in *Drosophila*. The widespread occurrence of needle-eye sperm in sterile hybrid males, paired with the potentially consistent point of spermatogenic failure (see Chapter 2), suggests that there may be an underlying, and potentially widespread mechanism for hybrid sterility, at least in the genus *Drosophila*. The genetic underpinnings for this phenotype could be studied across all other interspecies pairs in *Drosophila*.

In this study, no regions were identified as significantly enriched with *D. simulans* SNPs in males backcrossed to *D. mauritiana* possessing needle-eye sperm using PSIseq. I did, however, identify 10 regions that uniquely contained *D. simulans* SNPs and identified the genes associated with those regions (Table 5.2). A similar result was found for males backcrossed to *D. simulans* possessing needle-eye sperm.

### 5.4.1 An alternative theory to the genetic basis of hybrid sterility and Haldane's rule: meiotic drive

The unequal segregation of chromosomes during meiosis can result in an unequal representation of genes, their regulator elements or both, within a given population or species (Werren, 2011). Meiotic drivers are prevented from running rampant within a given population by the co-evolution of suppressors, especially since drivers can reduce the overall fitness of the individual (McDermott and Noor, 2010). For example, selfish elements on the X chromosome during sperm development may drive for an over-representation of X chromosomes *versus* Y chromosomes. The co-evolution of suppressor elements, usually arising on the autosomes (Hurst and Pomiankowski, 1991) in response to selfish elements establishes a genetic conflict between the selfish element and its suppressor. A hybrid individual derived from the mating of two separated

populations could possess a mismatch between the selfish element and the suppressors, resulting in dysfunction (Frank, 1991a). Although it was initially disregarded due to lack of empirical evidence, recent studies have suggested meiotic drive is a potential mechanism for the evolution of hybrid sterility (Phadnis and Orr, 2009; McDermott and Noor, 2010).

## 5.4.2 Could needle-eye sperm be linked to Dominance theory or meiotic drive?

### 5.4.2.1 Transporter proteins and hybrid sterility

As outlined in Table 5.1, only half the genes that were identified as potentially linked to needle-eye sperm in BCM have been characterized. Two of the genes, GD25754 and GD20661, have putative functions as transporter proteins. Previous studies, predominantly in mammalian sperm, have shown how transporters of ions and large molecules play a crucial role on sperm motility and overall fertility (Angulo et al., 1998; Touré et al., 2007; Maruyama et al., 2016). Although the importance of transporters in sperm motility has yet to be demonstrated in *Drosophila,* the gene *JYalpha*, linked to hybrid sterility between *D. melanogaster* and *D. simulans*, may function as a transporter protein (Masly et al., 2006; Flybase.org). *JYalpha* has a unique mechanism for the evolution of hybrid sterility whereby reproductive isolation is a by-product of a gene transposition event that causes some later generation hybrids to have two copies of the gene and others to have no copies of the gene (Masly et al., 2006). The potential role for transporters in sperm motility, and thus hybrid sterility, may be the supply and production of ATP to drive sperm tail movement (Mukai et al., 2004). Various sugar transporters are needed to drive glycolysis in sperm to produce ATP for flagellar movement (Mukai et al., 2004). The lack of a functional copy of *JYalpha* in some later-generation interspecies hybrids may thus cause the production of immotile sperm, underlying sterility.

The role of transporters in the formation of needle-eye sperm and how it relates to HR and hybrid sterility is not as obvious. These genes, if they are implicated in hybrid sterility, would require separate examination in their role with sperm tail function, instead of improper sperm head formation. Separately, the remaining five genes that are

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uncharacterized may be associated with spermatogenesis but additional inquiry into their molecular function is needed.

## 5.4.2.2    Notch signalling, spermatogenesis, and hybrid sterility

Based on the analysis of this study, no regions of the X chromosome were linked to the production of needle-eye sperm in BCM. In BCS, however, a small region on the X chromosome was identified to be enriched with *D. simulans* SNPs, which contained the signalling molecule, *Notch* (Duncan et al., 2016). Notch signalling has been implicated in *Drosophila* germ cell development (Assa-Kunik et al., 2007; Song et al., 2007; Kitadate et al., 2010). Cleavage of the cytosolic domain of Notch protein leads to the transcription of various Notch-targeted genes that have been implicated in cell differentiation (Borggrefe and Oswald, 2009).

In mammals, molecular components of the Notch signaling pathway have been found to be expressed at various stages of spermatogenesis, and aberrations of this pathway have been linked to male sterility (Hayashi et al., 2001; Hayashi et al., 2004; Garcia et al., 2013; Murta et al., 2013). Specifically in mice, *Notch1*, *Notch2*, and *DII4* are expressed in cells that are undergoing the early stages of meiosis. Furthermore, only *Notch1*, *Notch2*, and *DII4* are expressed in spermatids during the second meiotic division (Murta et al., 2013). The Notch pathway is highly conserved (Artavanis-Tsakonas et al., 1995) but may functionally operate at a species-specific level. As such, the introduction of a *D. simulans Notch* within the genetic background of *D. mauritiana* may provide a scenario wherein a foreign *Notch* may cause aberrations within the Notch signalling pathway, leading to aberrations in spermatogenesis. This hypothesis, however, would suggest that species specific Notch has the ability to disrupt an otherwise evolutionary conserved signalling pathway.

## 5.4.2.3    *Umbrea*, spermatogenesis, and hybrid sterility

In *D. melanogaster,* HP1-interacting protein (Hip) and heterochromatin protein 1 (HP1) are involved in the formation of heterochromatin and epigenetic gene regulation (Eissenberg et al., 1990; Schwendemann et al., 2008). Hip and HP1 form a protein complex with a newly evolved gene in *Drosophila, Umbrea,* and localize to pericentric

heterochromatin (Joppich et al., 2009; Ross et al., 2013). Umbrea, a by-product of a duplication event of *Heterochromatin Protein 1B* (*HP1B*; Levine et al., 2012; Ross et al., 2013), also localizes to the centromeres of developing spermatocytes (Ross et al., 2013). Mitotic errors, including delayed chromosomal alignment and lagging anaphase chromosomes, are seen in flies with knockdown *Umbrea* (Ross et al., 2013). This suggests that *Umbrea* plays an essential role in chromosome segregation. Interestingly, a severe reduction of *Umbrea* transcripts does not affect the location of Cid, a centromeric histone protein involved in the formation of the kinetochore during chromosome division in mitosis and meiosis (Blower and Karpen, 2001).

Centromeric proteins are under rapid evolution, alongside the associated heterochromatic repeats (Lohe and Roberts, 1988; Csink and Henikoff, 1998; Brideau et al., 2006; Malik and Henikoff, 2009), leading to the divergence of centromeric proteins across taxa (Cheeseman and Desai, 2008). It is likely that *Umbrea,* a centromeric protein, is under strong selective pressures alongside rapidly evolving centromeric DNA. If *Umbrea* undergoes rapid evolution within the genetic background of one population, it will only be compatible with the centromeric proteins that co-evolved under the same genetic background. The faithful execution of chromosomal segregation will, thus, rely on the appropriate and compatible interaction of the proteins involved. This is analogous to the meiotic drive hypothesis of hybrid sterility (McDermott and Noor, 2010).

If *Umbrea* is introduced into a foreign genetic background (e.g. from a separated and divergent population), then the sensitive system of chromosomal segregation becomes compromised. *Umbrea* in this example may be incompatible with other proteins involved in chromosomal segregation (e.g. the formation of the kinetochore), resulting in improper segregation. In the context of spermatogenesis, spermatocytes undergoing meiosis may not properly segregate (either at meiosis I, II, or both). This failure of chromosome segregation could account for the consistent pattern of the formation of needle-eye sperm seen across the multiple *Drosophila* interspecies hybrids examined in this study.

### 5.4.2.4    TAF1, spermatogenesis, and hybrid sterility

The transcription factor TFIID is a multiprotein complex composed of TATA-box binding proteins (TBPs) and TBP-associated factors (TAFs; Dynlacht et al., 1991; Walker et al., 2001; Matangkasombut et al., 2004). In *Drosophila,* the TAF1 isoform, TAF1-2, is abundantly localized to the testes, specifically within transcriptionally active pre-meiotic cells (Katzenberger et al., 2006; Metcalf and Wassarman, 2007). Further analysis found that TAF-1 co-localized to only paired X and Y chromosomes within spermatocytes (Metcalf and Wassarman, 2007). The pattern of TAF-1 localization has lead to the conclusion that TAF-1 assembles with other proteins to form a testis-specific TFIID complex that regulates transcription during spermatogenesis (Chen et al., 2005; Metcalf and Wassarman, 2007), yet its role in the pairing of X and Y chromosomes has not been further explored.

Intergenic rDNA regions that are found within the X heterochromatin and Y centromere are involved in X-Y pairing in *Drosophila* (Mckee et al., 1992). These regions, which consist of a 240bp repeat, differ among closely related species of the *D. melanogaster* subgroup (Lohe and Roberts 1990). Disruptions in X-Y pairing are observed when this repeat region is deleted, resulting in X-Y nondisjunction during meiosis (Mckee et al., 1992). X-Y misparing leading to sterility has also been previously observed in mammalian interspecies hybrids, often leading to degeneration of spermatocytes (Matsuda et al., 1991; Ishishita et al., 2015). Since TAF-1 is associates with the paired X and Y chromosomes, it is possible that it is specifically associated with the rDNA repeat regions of the X and Y chromosome and plays a role in X-Y pairing. If so, TAF-1 may be under the same selective pressures to rapidly evolve alongside the associated rDNA, making it only functionally compatible within the genetic background it has evolved under. If introduced into a foreign genetic background, as similar scenario (as described with *Umbrea*) would result in genetic incompatibility.

### 5.4.3    The potential role of X chromosome in hybrid sterility

Numerous lines of evidence suggest that the X chromosome plays a large role in interspecies hybrid sterility (Coyne and Orr, 1989; Guenet et al., 1990; Storchova et al.,

2004; reviewed in: Coyne and Orr, 2004). In *Drosophila,* X-linked substitutions have a greater impact on hybrid sterility than autosomal substitutions (Coyne and Orr, 1989). Furthermore, the genes that have so far been identified as linked to hybrid sterility in interspecies *Drosophila* hybrids have been found on the X chromosome (Sun et al., 2004; Phadnis and Orr, 2009).

This brings into question why only one region in BCS was linked to the X-chromosome and there was no X effect in BCM (Table 5.1). First, it is possible that PSIseq failed to identify any significant regions for the same reason that it failed to identify X-linked regions. Second, the large X-effect seen in previous studies may reflect the assays used to characterize fertile hybrids from sterile hybrid in *Drosophila.* Normally, hybrid sterility is characterized by the lack of sperm motility (e.g. Perez et al., 1993; Phadnis and Orr, 2009; Masly et al., 2006; Dickman and Moehring, 2013). Although this is a useful assay to uncover genes for hybrid sterility, it may too broad. Numerous stages of spermatogenesis can lead to non-motile sperm, thus using motility as an assay for hybrid sterility may lead to a wide capture of different genetic regions. The use of needle-eye sperm as the phenotype for hybrid sterility in *Drosophila* may narrow the possible genetic regions. Finally, the needle-eye phenotype may only be a by-product of autosomal gene incompatibility, with no actual link to the X-chromosome. This may be the most plausible scenario for why the X-chromosome was not implicated in the formation of needle-eye sperm.

### 5.4.4 Why PSIseq was not able to identify introgressed regions of *D. simulans* or *D. mauritiana* in backcrossed males with needle-eye sperm

As described in the Materials and Methods section, males were pooled into a single sample based on their backcross lineage (i.e. BCS or BCM) and whether the male produced needle-eye sperm or fertile sperm. The purpose of pooling individuals into one of the four above groups was based on financial constraints of the project and, more importantly, the strong 50:50 phenotypic segregation noted each generation, even after 10 generations of backcrossing to either *D. simulans* or *D. mauritiana.* Males produced from

the same mother were either fertile males (single and motile sperm) or sterile males (needle-eye sperm). Here, a consistent ratio or 50:50 fertile *versus* sterile males were noted in all BCS10 or BCM10 males produced from a single mother. This strongly suggests that a single Mendelian region is responsible for needle-eye sperm. As such, pooling individuals was predicted to not affect the identification of the region that contributes to needle-eye sperm; rather it was expected that pooling would help eliminate any potential regions that, by chance, happen to also be present after 10 generations of backcrossing, but did not contribute to needle-eye sperm and would be only present in some of the backcross lineages. It is possible, however, that pooling individuals may have disguised multiple regions that would contribute to needle-eye sperm. If many genes individually contribute to needle-eye sperm, then pooling individuals within a single sample could mask individual genes and thus PSIseq would not be capable of detecting regions of *D. simulans* or *D. mauritiana* introgression.

Although I was unable to use PSIseq to identify statistically significant regions of either *D. simulans* or *D. mauritiana* in BCM or BCS, respectively, I was able to identify regions that did have *D. simulans* or *D. mauritiana* SNPs that were exclusive to males producing needle-eye sperm. There may be detectable regions that contribute to needle-eye sperm, but perhaps the use of PSIseq is not appropriate for the experiment conducted in this study. However, it may also be possible to revisit this study without pooling individuals into a single sample and re-analyzing using PSIseq. Traditional mapping techniques, such as microsatellite mapping, may also be useful in uncovering regions responsible for needle-eye sperm. Regardless, the segregation of 50% sterile *versus* 50% fertile in BCS and BCM males does suggests needle-eye sperm is produced by a single locus of strong phenotypic effect, and can thus potentially be genetically mapped to a single region.

Needle-eye sperm appears to be a universal phenomenon within interspecies hybrids in *Drosophila.* As such, uncovering the genetic factors that give rise to needle-eye sperm could provide a universal mechanism for hybrid sterility in *Drosophila.* The strong bimodal segregation of sterile *versus* fertile males after backcrossing to either *D. simulans* or *D. mauritiana* for 10 generations suggests that this phenotype can likely be induced by a single sufficient genetic region, making it feasible to identify its genetic

underpinnings. Thus, future analyses to refine the experimental design of this study would be worthwhile.

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# Chapter 6

# 6     General Discussion

## 6.1    Thesis summary

There is little empirical evidence on what is the genetic basis of hybrid sterility. One of the most common observations seen across many interspecies hybrids is the high susceptibility to sterility in the heterogametic sex (e.g. males in *Drosophila*) over the homogametic sex (Haldane, 1922). This phenomenon, termed Haldane's rule, indicates that there may be similar genetic underpinnings that would promote the formation of sterile hybrids in diverging populations. As such, identifying at what stage spermatogenesis fails across multiple interspecies pairs in *Drosophila* will provide insight into the possible genetic mechanisms that lead to sterility.

Here, I identify for the first time the specific stage at which spermatogenesis fails in sterile male hybrids in a genus wide study in *Drosophila.* The studies presented in this dissertation identify common cellular trends among three interspecific sterile male hybrids. Most importantly, a previously uncharacterized sperm phenotype was identified across all hybrid males studied, which I call "needle-eye" sperm.

Overall, abnormal testes morphology with visibly disorganized sperm bundles is seen across all interspecies pairs examined (Chapter 2). At meiosis I, non-disjunction is apparent in all sterile hybrids studied, leading to half the number of sperm produced after spermatogenesis (Chapter 2). In addition, sperm have two tails per nuclei for two interspecies studied (Chapter 2). Nuclei length in aged (6 days old) sterile male hybrids exhibit rapid decondensation compared to young (1 day old) sterile male hybrids (Chapter 2). Furthermore, sperm nuclei of young sterile males differed in length compared to young males from the parental species, exhibiting either decondensed nuclei or more condensed nuclei (Chapter 2). This mis-packaging of sperm nuclei is not a by-product of a loss of bi-allelic protamine expression, as both parental protamine alleles are expressed in sterile male hybrid in one interspecific cross of *Drosophila* (Chapter 4). Although hybrid males possess meiotic and sperm morphology defects, sperm produced

are alive and have active mitochondria (Chapter 3). This finding is unexpected because mitochondria do not differentiate into major and minor derivatives in sterile males (Chapter 2), yet they appear to maintain their functionality. Finally, all interspecific hybrid males studied possess a previously uncharacterized sperm phenotype, wherein two nuclei appear joined. This novel sperm phenotype was called "needle-eye" sperm and its genetic basis was identified in one interspecies cross as being linked to centromeres and telomeres (Chapter 5). Together, the evidence presented in this dissertation suggests that failures during meiosis I of spermatogenesis, likely due to divergence at sequences near the centromeres or telomeres, results in the formation of needle-eye sperm, and thus the sterility observed in hybrid males in *Drosophila.* Furthermore, common cellular trends observed in a wide variety of interspecies pairs in *Drosophila* suggests a similar genetic basis, which would run counter to the proposed random genetic mutation mechanism of the Bateson-Dobzhansky-Muller incompatibility model (BDM model; Bateson, 1909; Dobzhansky, 1934; Muller, 1940).

## 6.2   Meiotic errors as the cause for hybrid sterility in *Drosophila*

The earliest stage at which spermatogenesis fails was identified during meiosis I in all interspecies crosses studied (Figure 2.1, 2.2, 2.3). After anaphase I, it was evident that a non-disjunction event occurred, resulting in the two daughter cells possessing unequal chromosomes, with two interspecific crosses exhibiting chromosomal bridges (Figure 2.2, 2.3). Previous cellular analyses of sterile hybrid male testes in *Drosophila* did not focus on the presence or absence of mis-segregated chromosomes (Kulathinal and Singh, 1998; Hardy et al., 2011); however, chromosomal bridges and chromosomal mis-segregation has been observed in interspecific sterile hybrids in plants and in inviable hybrids in *Drosophila* (McClintock, 1933; Beasley, 1941; Ferree and Barbash, 2009). The effects of chromosomal mis-segregation during meiosis I could result in the improper individualization of sperm cells during spermiogenesis. As such, mis-segregation during meiosis I may result in a cascade of defects in later stages of spermatogenesis, thus accounting for the reduction to roughly half the number of sperm produced after spermatogenesis in sterile hybrid males (Figure 2.4). Errors during meiosis, leading to additional downstream defects in spermatogenesis has previously demonstrated in

*Drosophila melanogaster* (Casal et al., 1990). Thus mis-segregation of chromosomes may affect proper sperm individualization, which could explain the presence of the needle-eye sperm head phenotype (Chapter 5). Another possible mechanism for the reduction of sperm produced is that errors in meiosis may trigger apoptosis of sperm cells, as genes involved in apoptosis in *Drosophila* also play a role during spermiogenesis (Huh et al., 2004). This possibility, however, is unlikely since cross-sectional analyses using Transmission Electron Microscopy (TEM) of hybrid male testes reveal that sperm nuclei possess two tails (Figure 2.1, 2.3; Hardy et al., 2011). As such, defects in meiosis I are more likely to affect sperm individualization during spermiogenesis than triggering sperm apoptosis.

A novel sperm phenotype seen across all interspecies crosses studied in this dissertation is the conjoined sperm heads, denoted as "needle-eye" sperm (Figure 5.1). Mis-segregation of chromosomes during meiosis, as seen in Chapter 2, has been speculated to affect individualization of sperm cells and in addition, may lead to the formation of this needle-eye sperm. To my knowledge, this sperm phenotype for sterile hybrid males in *Drosophila* has not been previously reported in any interspecies hybrids. As such, uncovering the genomic regions that give rise to needle-eye sperm in sterile hybrid males could identify the genetic basis of hybrid sterility in *Drosophila.*

Although no significant regions were implicated in the formation of needle-eye sperm in sterile hybrid males of *Drosophila simulans* mated to *Drosophila mauritiana,* non-significant regions that were uniquely enriched with either *D. simulans* or *D. mauritiana* SNPs were identified (Chapter 5). Two genes were identified as candidate *D. mauritiana* male sterility genes when present within a *D. simulans* genetic background (Table 5.1, Chapter 5): *GD25754* and *GD20661.* These genes have been given a putative role as transporters, suggesting that they may affect sperm tail function (see discussion in Chapter 5). Interestingly, two candidate *D. simulans* sterility genes within a *D. mauritiana* background play a role in chromosome interactions during division (see discussion in Chapter 5). These genes lend further support to the theory of Meiotic Drive in the formation of sterile hybrid males in *Drosophila* and thus should be investigated further.

Lastly, it is possible that there may be no individual gene or genes that are involved in the formation of sterile hybrid males, but rather non-genic regions (e.g. centromeres) play the most prominent role. This will be discussed further below.

## 6.3   Mitochondrial function and hybrid sterility

Mitochondrial function and activity plays a direct role in sperm motility (Ruiz-Pesini et al., 1998). Although the derivation of mitochondria into the sperm tail is not the initial stage at which spermatogenesis breaks down among interspecies hybrids examined in this dissertation (Chapter 2), a breakdown in mitochondrial function could represent a downstream cascade defect. During spermiogenesis, specifically when Nebenkern form, mitochondria fuse and differentiate into two derivatives, the major and minor derivatives (Tokuyasu 1974; Hales and Fuller 1997). In addition, genes involved in mitochondrial formation along the elongating sperm tail have been implicated in male sterility (Hales and Fuller 1997; Greene et al., 2003; McQuibban et al., 2006; Riparbelli and Callaini, 2007), although these genes have not been examined from the context of interspecific hybrid male sterility. In Chapter 2, the interspecific sterile hybrid males studied all exhibited mitochondria that did not properly differentiate into the major and minor derivatives (Figure 2.1G, 2.3G; Hardy et al., 2011). As such, I speculated that interspecific sterile hybrid males may exhibit dysfunctional mitochondrial activity. Surprisingly, mitochondrial membrane potential, an appropriate assessor of mitochondrial activity (Pendergrass et al., 2004), was not reduced in interspecific hybrids compared to parental males (Chapter 3). This suggests that the lack of differentiation and development of the major and minor mitochondrial derivatives does not affect mitochondrial function, at least in terms of membrane potential. To date, the function of each derivative remains unclear, but some theories have been postulated, including a species specific influence on the wave-like movement of the sperm tail (Tokuyasu, 1974). If this is true, it is possible that interspecific hybrid males in *Drosophila* possess immotile sperm due to the failure to establish both derivatives. As such, the genes involved in the differentiation process of mitochondria could provide some insight into this postulation.

## 6.4  Protamines and hybrid sterility

Sperm nuclei measured in each interspecies hybrid males studied in this dissertation appear improperly packaged (Figure 2.5). Sperm nuclei of hybrid males appeared shorter (more condensed) or longer (decondensed) compared to males of parental species (Figure 2.5, 2.6, 2.7, 2.8, 2.9). These results suggest improper DNA packaging, as well as protamine mis-expression, which has been previously observed in *D. simulans* and *D. mauritiana* hybrids (Moehring et al., 2007). In *Drosophila,* it has been shown that due to the temporal expression of protamines, both parental alleles are expressed within the offspring produced (Jayaramaiah-Raja and Renkawitz-Pohl, 2005; Kanippayoor and Moehring, 2012). As such, I proposed that interspecific sterile hybrid males may suffer from incorrect allelic expression of protamines, leading to improper packaging of nuclear material in sperm heads. Sterile hybrid males formed from the interspecies cross between *D. simulans* and *D. mauritiana* also exhibited bi-allelic protamine expression, similar to the parental species (Figure 4.3), thus ruling out a lack of bi-allelic expression of protamines as a cause of sterility in hybrid males in *Drosophila.*

The improper nuclei packaging observed in all interspecies sterile hybrid males examined in this dissertation may have resulted from several possible mechanisms. First, protamines are expressed from both alleles, but are expressed at incorrect levels in sterile hybrid males, an observation previously noted in hybrids produced from *D. simulans* and *D. mauritiana* (Moehring et al., 2007). Interestingly, protamine mis-expression was not observed in hybrids produced between *Drosophila pseudoobscura pseudoobscura* and *Drosophila pseudoobscura bogotana,* which may relate to the phenomenon that aged hybrid males regain partial fertility (Phadnis and Orr, 2009; Gomes and Civetta, 2015). Further analysis of the age related effects on sperm nuclei length observed in Chapter 2 in relation to protamine expression could provide insight to why nuclei in sterile hybrids were either more condensed or decondensed compared to the parental species.

Another possible, although less likely, explanation for the variation in sperm nuclei length in sterile male hybrids is the incompatible interactions between each parental protamine. As a by-product of hybrids expressing protamines from two different species,

protamines may not properly bind to DNA or properly link to neighbouring protamines through the appropriate amino acid residues involved in these interactions (reviewed in: Kanippayoor et al., 2013). The only caveat to this theory is that protamines have been shown to be highly conserved across many species within the *Drosophila* genus (Alvie et al., 2013), and thus are not likely divergent between closely-related species, such as those examined within this dissertation.

Finally, the pathway that results from the transition of histone-base to protamine-base nuclear organization may be compromised within interspecific sterile hybrid males. The disassociation and degradation of the transition protein, $Tpl^{94D}$, allowing for protamines to properly bind to chromatin (Rathke et al., 2010) may be effected in sterile hybrid males, resulting in improper packaging of hybrid sperm nuclei. As the pathway involved in the movement from histone to protamine remains unclear in *Drosophila,* it's possible that the genes involved are not conserved like protamines and require a species-specific network in order to properly function. Therefore, understanding all key players involved in packing of sperm nuclei and their evolution across species would provide insight into the mis-packaging of nuclear material in sperm of sterile hybrid males.

## 6.5 Genetic basis of hybrid sterility in *Drosophila*

The findings observed in the studies of this dissertation identified notable trends across all interspecies crosses studied. In Chapter 2, meiotic failures occur during meiosis I, leading to mis-segregation of chromosomes and roughly half the number or sperm produced after spermatogenesis. Furthermore, each sperm nucleus appears to have two tails with no differentiation of mitochondrial derivatives (Chapter 2). Finally, a novel sperm phenotype has been identified in all interspecies hybrids studied in this dissertation, denoted as "needle-eye" sperm, as sperm heads appear paired (Chapter 5). These general trends, in addition to all other trends observed in this dissertation, do not provide support for the most acknowledged model for the genetic basis of hybrid sterility and Haldane's rule, the Bateson-Dobzhansky-Muller Incompatibility model (BDM model; Bateson, 1909; Dobzhansky, 1934; Muller, 1940). The BDM model describes sterility as a product of random, genetic incompatibilities in a hybrid male (Bateson,

1909; Dobzhansky, 1934; Muller, 1940). As such, it would be expected that sterility phenotypes would differ across multiple interspecies crosses, with few instances of similar phenotypes in hybrid breakdown. This, however, is not seen in sterile hybrid males produced in the genus *Drosophila*. Stages of spermatogenic breakdown are similar across all three interspecies pairs observed, with some maternally related exceptions (Kulathinal and Singh, 1998; Hardy et al., 2011). As such, two possible conclusions could be drawn. First, the BDM model does not apply to hybrid sterility in *Drosophila*, and a common genetic basis can be identified in all interspecies hybrids in this genus, leading to similar sterility phenotypes observed in this dissertation. Second, the BDM model does apply to hybrid sterility in *Drosophila*, and random genetic changes lead to similar incompatibilities within interspecific hybrids due to certain stages of spermatogenesis being more susceptible and sensitive to genomic changes. In this latter scenario, the cellular basis of hybrid sterility in *Drosophila* reveals trends among hybrids, but the underlying genetic basis would be random.

Alternatively, if there is indeed a common genetic basis, the results presented in this dissertation provide some evidence for meiotic drive, a theory that was previously refuted (Coyne and Orr, 2004), but has regained some interest, in light of recent findings (reviewed in: McDermott and Noor, 2010). Meiotic drive suggests that selfish genetic elements are co-evolving alongside suppressors within a population, presenting a contained system that, upon disruption due to a hybridization event, uncovers the selfish element (McDermott and Noor, 2010). Specifically, X-linked selfish drivers, which have a higher occurrence (Hurst and Pomiankowski, 1991), that affect fertility, or disrupt sex ratios, promote the evolution of suppressors to contain their deleterious effect on the population (Edward, 1961; Crow, 1991). As such, the break-up of the selfish driver from its suppressor(s) due to a hybridization event, may unleash the deleterious effects of the driver, affecting fertility of the individual. In *Drosophila*, it has been previously demonstrated that a gene, *Overdrive*, gives rises to both hybrid male sterility and segregation distortion between two subspecies of *Drosophila pseudoobscura: Drosophila pseudoobscura pseudoobscura* and *Drosophila pseudoobscura bogotana* (Phadnis and Orr, 2009). This finding is especially important, as *D. p. pseudoobscura* and *D. p.*

*bogotana* represent young species that have not accumulated as many genetic differences as older species (Matute et al., 2010). In addition, the genetic basis of hybrid sterility between *D. p. pseudoobscura* and *D. p. bogotana* reveals that meiotic drive may be an evolutionary force for species isolation (Phadnis and Orr, 2009). Furthermore, although this is highly speculative, two possible genes were identified as possible candidates in the formation of needle-eye sperm in sterile hybrid males backcrossed to *D. mauritiana* (Chapter 5). Both *Umbrea* and *Taf1* have been linked to chromosome segregation and pairing, respectively, during meiosis (Metcalf and Wassarman, 2007; Ross et al., 2013).

It is possible that genes may not give rise to the formation of sterile hybrid males in *Drosophila;* instead, non-genic regions, such as centromeres, may play a larger role. Heterochromatic centromeres recruit the segregation machinery during meiotic division (Dernburg et al., 1996). Centromeres are rapidly evolving alongside the proteins that are associated to them (Csink and Henikoff, 1998) due to strong adaptive pressures, potentially caused by the selfish drive of centromeres to be incorporated into the pronucleus of the egg (Henikoff et al., 2001). The rapid evolution of centromeres has lead to high sequence divergence between closely related species (Lohe and Roberts, 1998). Thus, identifying the sequences involved in the mis-segregation of chromosomes during meiosis I in all interspecies crosses studied in this dissertation could also provide insight into the mechanism of how this stage of spermatogenesis fails.

## 6.6   Concluding remarks

In conclusion, general phenotypic trends of sterile hybrid males in *Drosophila* suggest a common genetic basis. Most importantly, meiosis I has been identified as the earliest stage of spermatogenesis to fail across all interspecies hybrids studied in this dissertation, a finding that has never been previously observed. In addition, I have identified a new sperm phenotype in all interspecies hybrid males studied, wherein two sperm heads are fused together. The findings presented in this dissertation and the conclusions drawn here require further exploration to elucidate how, genetically, Haldane's rule relates to the evolution of hybrid sterility in *Drosophila.* The sequences involved in the mis-segregation of chromosomes during meiosis I must be identified to describe, specifically,

how meiosis I fails in sterile hybrid males. Further fine-mapping of the regions implicated in the formation of needle-eye sperm, identified in Chapter 5, will identify possible candidate genes or genomic structures that can be tested. Additional questions that require investigation involve understanding the downstream cascade defects that arise during spermatogenesis. For example, how protamines are misregulated in sterile hybrid males, resulting in the deviation from normal sperm nucleus length. Also, additional exploration of how parasperm and eusperm are differentially affected in terms of hybrid sterility between *D. p.pseudoobscura* and *Drosophila persimilis* (Chapter 2) would provide some insight into whether parasperm and eusperm operate on different molecular pathways and are, thus, under different evolutionary pressures. Finally, although this was not addressed in this dissertation, certain interspecific crosses exhibit maternally related sterility phenotypes that did not fit into the general trends observed in this dissertation (Kulathinal and Singh, 1998; Hardy et al., 2011). In the case of a female *D. mauritiana* mating with either a male *D. simulans* or *Drosophila sechellia,* hybrid sons do not produce sperm and spermatogenesis appears to arrest prior to meiosis (Kulathinal and Singh, 1998). As such, these hybrids present an interesting and unique scenario, wherein the maternal genome, or just the X chromosome, appears to play a role in how sterility manifests, a potentially important evolutionary force shaping postzygotic barriers. Overall, our understanding of how Haldane's rule and hybrid sterility arises between diverging populations remains limited. However, the results observed here provide new evidence for how hybrid sterility arises in *Drosophila,* which I hope prompts a new direction for further research in this field.

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# Appendices

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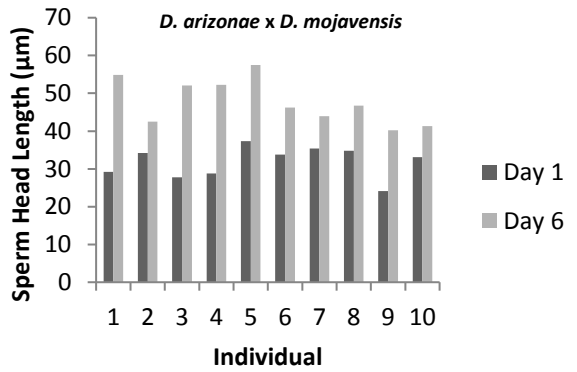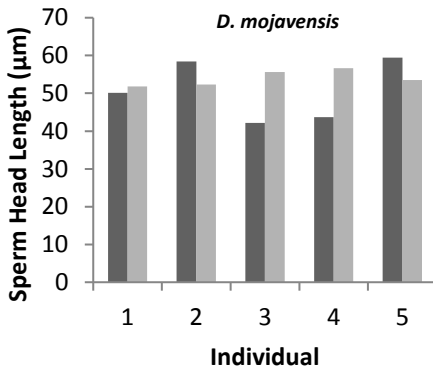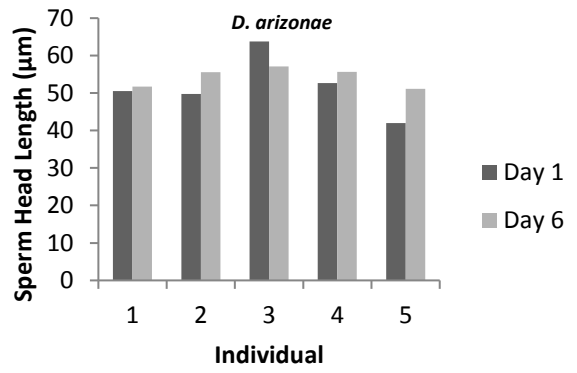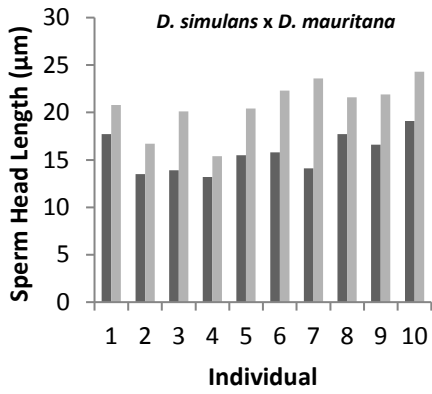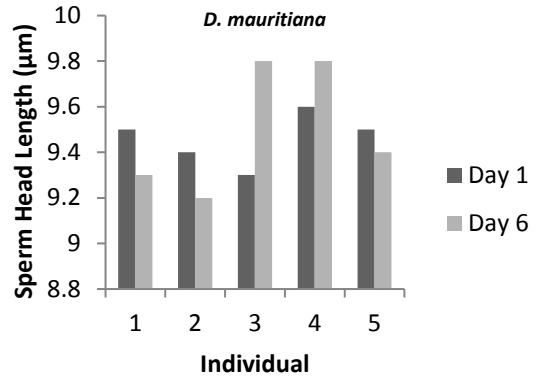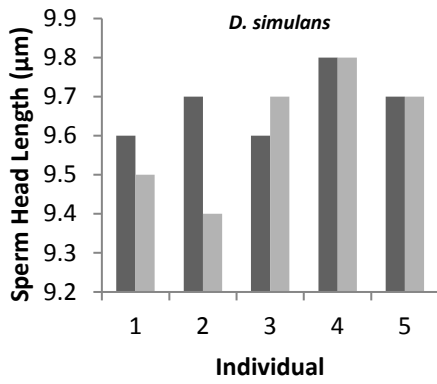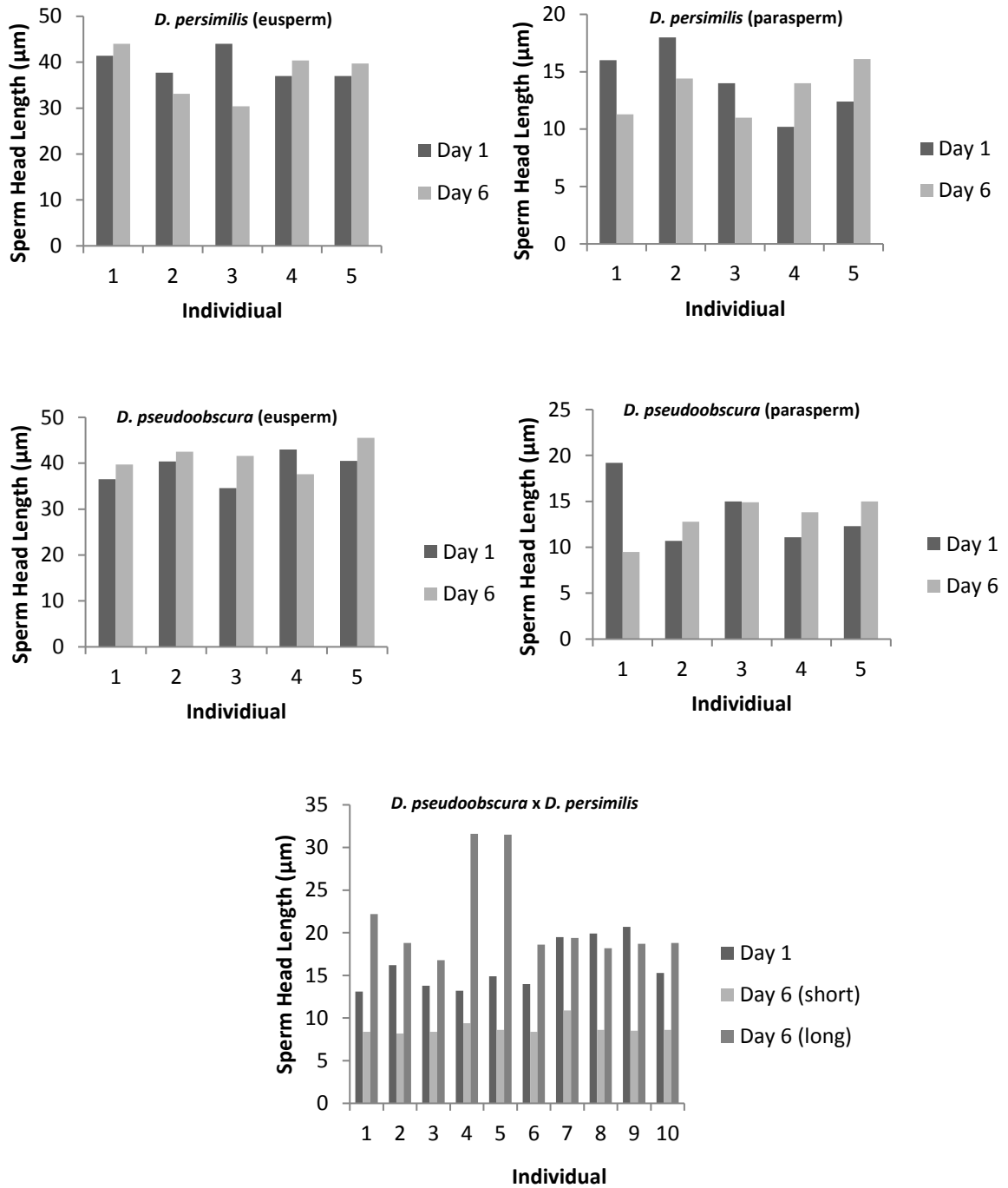
Supplementary Figure 1: Histographs for individual sperm nuclei length of parental species and their interspecies hybrids.

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