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Reproductive Biology and Speciation in Drosophila pseudoobscura

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Graduate Program in Biology

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

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By

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Abstract

Sexual selection is an important force driving the evolution of reproductive traits, including sperm morphology and mating behaviour. Divergent sexual selection among populations can eventually lead to errors in spermatogenesis in inter-population hybrids, and subsequently speciation. In Chapter 2, I identify a novel sperm class and how its proportion in the ejaculate is adjusted when *Drosophila pseudoobscura* males are exposed to competition. In Chapter 3, I assess how competition causes both males and females to adjust their mating behaviour. In Chapter 4, I characterize interspecific hybrid spermatogenic breakdown from two closely-related sub-species. While the genetics of hybrid sterility has been widely studied, the defective spermatogenic phenotypes have largely been ignored. I found that spermatogenic errors are exclusively postmeiotic and partially caused by divergence at the 'speciation gene' *overdrive*. The results of this thesis expand our understanding of the evolution of novel reproductive traits and the evolution of hybrid male sterility.

Keywords: Evolution, Sexual Selection, Sperm Competition, Cryptic Female Choice, Parasperm, Speciation, Spermatogenesis, *Drosophila*, *pseudoobscura*,

Statement of Co-Authorship

Chapter 2

Alpern, J.H.M., and Moehring, A. J.

J.H.M.A. discovered the phenotype. J.H.M.A. and A.J.M. designed the study. J.H.M.A. performed all experiments except the dissection of female reproductive tracts, performed by A.J.M. J.H.M.A. analyzed the data. J.H.M.A. and A.J.M. wrote this chapter.

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List of Abbreviations

ANOVA: Analysis of Variance

BDM : Bateson-Dobzhansky-Muller Model

BOG: *Drosophila pseudoobscura bogotana*

CHC: Cuticular Hydrocarbon

CPC: Cyst Progenitor Cells

ct: *cut* (GA23816)

DAPI: 4',6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

F_1^{BOG}: F_1 Hybrids between *Drosophila pseudoobscura pseudoobscura* males and *Drosophila pseudoobscura bogotana* females

GPC: Germline Progenitor Cells

HSC: Haploid Spermatocytes

JAK-STAT: Janus kinase/signal transducers and activators of transcription pathway

Kb: kilobases

ll: *lanceolate*

MSCI: Meiotic Sex Chromosome Inactivation

ovd: *overdrive*

PBS: Phosphate buffered saline

Prdm9: *Positive regulatory domain zinc finger protein 9*

PSC: Primary Spermatocytes

PSG: Primary Spermatogonia

QTL: Quantitative Trait Loci

SDSC: San Diego Stock Center

se: *sepia* (GA19859)

sp: *snapt*

tt: *tilt*

USA: *Drosophila pseudoobscura pseudoobscura*

Chapter 1

Introduction to Sperm Competition and Reproductive Isolation in *Drosophila pseudoobscura*

1.1 Parasperm Function in Fertilization Success

1.1.1   Postcopulatory Sexual Selection & Sperm Morphological Evolution

Sexual selection is a process whereby variation in sexual characteristics influences mating success. In most species, sexual selection acts predominantly on males (1, 2). Methods of enhancing mating success benefit males by granting them access to more female mates, increasing the number of sired offspring (3). In species where females mate with only one male, sexual selection is expected to be exclusively precopulatory. Precopulatory sexual selection acts upon a male's attractiveness to a female or his physical competitive capacity against other males (4). However, selection for fertilization success does not end at mate acquisition. Postcopulatory sexual selection exists when females influence paternity after mating (cryptic female choice) or when there is competition for fertilization amongst ejaculates of at least two males for (sperm competition)(5).

In polyandrous species, the effects of postcopulatory sexual selection are predicted to produce rapid evolution of male traits that promote ejaculate success (2, 5, 6). There are numerous ejaculate traits that are strongly correlated with the amount of sperm competition a species experiences. For example, the amount of sperm competition within

species of deer mouse correlates strongly with the shape, angle, and size of the hook present on the sperm, suggesting sperm competition has played a critical role in the evolution of deer mouse sperm morphology (7). Several species of polyandrous deer mice have a hook present at the apical tip of sperm heads that is used to produce sperm aggregates. In one species, these sperm aggregates are only formed among the sperm of the same male (sperm from other males is competitively excluded) and allow the sperm to act together to propel more rapidly through the female reproductive tract, thus increasing that male's chance of fertilizing the egg in the presence of sperm competition (8).

1.1.2   Sperm Heteromorphism: Eusperm and Parasperm

Organisms across many taxa have evolved heteromorphic sperm in response to postcopulatory sexual selection (9–11). In these species, there exist at least two sperm morphs: fertilizing eusperm and sterile parasperm. It is believed that the sterile sperm are crucial in increasing fertilization success, although the precise role parasperm play in reproduction has remained elusive, mainly due to limits in experimental procedures (9). Interestingly, in some species, parasperm appear simply as short eusperm, while in others, the parasperm have evolved unique morphological or cellular characteristics (12, 13). Some of these morphological characteristics have been used as hallmarks to determine parasperm function (14).

Several theories exist to explain how non-fertilizing sperm may function, yet most of the theories have limited or absent experimental support (15, 16). In some taxa, it has been suggested that parasperm have evolved to aid eusperm along the reproductive path

to the eggs. A few externally fertilizing marine organisms show decreased diffusion of eusperm on the path towards the eggs compared to what is expected based on fluid dynamics (13, 17). It is believed that sperm competition could also drive the evolution of parasperm (6, 18). These competitive mechanisms have been classified into offensive competitive measures (kamikaze sperm) and defensive competitive measures (blocking sperm)(19, 20). The kamikaze sperm hypothesis has garnered theoretical attention, yet no experiment has revealed the presence of suicidal sperm. Some speculate that the presence of proteolytic activity within parasperm could function in breaking down competing sperm; however, proteolytic activity in sperm is typically low (17).

The potential for sperm to function as blockers against competing sperm has been tested in a few *Drosophila* species which contain morphologically distinct parasperm (11, 21). In these species, sperm that do not successfully access female long-term sperm storage organs are eliminated from the reproductive tract. Eusperm that successfully make it to the storage organs are held until fertilization. For parasperm to function as blockers, they must successfully access the sperm storage organs and stay in sperm storage until encountering competing ejaculates (22). In all experiments conducted to date, the parasperm manage to enter sperm storage, yet they are reduced drastically in numbers before encountering a competing ejaculate, suggesting they are unlikely to function as blockers (9, 21, 23).

In *Drosophila*, only a small subset of species known as the *obscura* group have morphologically distinct parasperm (11, 24). One member of this subgroup, *D. pseudoobscura*, has served as a major model for assessing parasperm function (23, 25, 26). In this species, the eusperm are four times longer than the parasperm (23). The

parasperm are believed to be a male adaptation to cryptic female choice as they protect

eusperm from spermicide compounds produced in the reproductive tract of the female

(25, 27). Traditionally, *D. pseudoobscura* were thought to deposit a single type of

parasperm into the female reproductive tract. However, the parasperm show a large,

potentially bimodal size distribution, producing uncertainty as to whether or not there

exists one or two parasperm morphs in this species (23). This has potentially prevented an

accurate assessment of the role of parasperm in this species.

In Chapter Two, I present the characterization of two distinct classes of parasperm

in *D. pseudoobscura*. Using this new classification of two distinct parasperm, I identified

the role of each sperm class in fertilization. I hypothesized that if the proportion of one

parasperm class present in the ejaculate correlates with the survival of eusperm within the

female reproductive tract, then this would indicate a role in protection from female

spermicides. I further hypothesized that if the proportion of the other parasperm class

would increase when other males were present, then this would indicate a role in sperm

competition. I predicted and showed that cryptic female choice and sperm competition,

the latter of which has been largely ignored, has played a role in the evolution of

parasperm in this group.

1.2 Evolution of Plasticity in Response to Sperm Competition

Traits that evolve in response to competition can require a large energetic

investment (28, 29). Production of such traits will produce an energetic trade-off. A

classic example of this evolutionary trade-off is the evolution of interspecific variation in

ejaculate size that is positively correlated with sperm competition (30–32). These larger

ejaculates are composed of more sperm per ejaculate, and require larger testes to

accommodate the increase in spermatogenesis. As a result of this trade-off, many of the

species with larger ejaculates have a delay in sexual maturity to allow more time for

testicular growth (33).

The interspecific difference in testicular size is an example of a fixed response to

sperm competition, as this phenotypic difference can be genetically determined during

development (30, 31, 34, 35). In contrast to fixed traits, some are variable depending

upon the amount of competition that is present (36–42). In a natural environment, levels

of sperm competition are expected to fluctuate regularly; not every copulation event

exposes a male's ejaculate to the same number of competing sperm. Furthermore, the

attributes that evolve under competition may be neutral or deleterious to fertilization or

the male's fitness in the absence of competition. As a result, there is predicted to be

strong selection for the evolution of phenotypic plasticity for traits conferring a

competitive advantage if they impose a fertility or energetic cost in the absence of

competition (6, 43). Males are predicted to assess their environment and tailor their

ejaculate characteristics according to the potential of experiencing competition within the

female reproductive tract. There is a large body of empirical support for the presence of

plasticity for ejaculate characteristics in mammals and insects. In *Rattus norvegicus*

(Norway rats), *Microtus pennsylvanicus* (meadow vowles), *Pieris napi* (green-veined

white butterfly), and *Nephila edulis* (orb-web spider) males ejaculate more sperm into a

female in the presence of competitor males at mating (44–47). In *Danaus plexippus*

(monarch butterfly) and *Pieris rapae* (small white butterfly), males assess the number of

males with whom the female has mated, ejaculating greater amounts of eusperm when

females have mated with a greater number of males (48, 49). In the moth *Plodia interpunctella*, males will ejaculate more sperm into the female when there are greater numbers of sperm already present in her reproductive tract (50).

In *Drosophila melanogaster*, a species of fly without parasperm, males respond to the number of competitor males with whom a female has mated by increasing mating duration (51). This results in increased sperm transfer, much like in mammals and butterflies. Exposure to potential mating rivals also increases mating duration, increasing the amount of seminal fluid proteins that are transferred to the female (52–54). These seminal fluid proteins are believed to increase the viability of the sperm transferred, thus increasing paternity share and competitive capacity. In several other species of *Drosophila*, males also increase mating duration in response to competing males, though this response does not appear to be ubiquitous across the genus (55, 56). Although untested, it is believed that all *Drosophila* species with increased mating duration also increase the amount of sperm protein transferred (51-54).

Males use multiple cues to determine the presence of competing males. In mammals and insects, pheromonal cues produced by rivals are critical in eliciting a plastic response in sperm composition (45, 46, 57). In *Drosophila*, sound, touch and pheromones of potential competitors are sufficient to elicit a plastic response if present in pairs, though none are sufficient to produce the response alone (58). The underlying mechanisms of sound and pheromone production and detection are rapidly evolving, and therefore are different between even closely related species (59–62). Since it would be maladaptive to respond plastically to males that are merely pseudorivals (do not pose a fertility risk), it is predicted that the response to heterospecific rivals should be weak (26).

Understanding whether or not males respond to heterospecific pseudorivals would provide strong insight into the evolution of cues utilized by males to detect real rivals.

In *D. pseudoobscura*, males do not appear to respond to the presence of distantly-related heterospecific pseudorivals; however closely related species have never been assessed (26). In Chapter Three, I address the evolution of plasticity in the face of sperm competition by exposing males to rivals from distantly and closely related species. I hypothesized that if the evolution of signals indicating the presence of males is gradual, then distantly related species would not produce a response, while more closely related species would produce a weak response. I predicted that when males of *D. pseudoobscura* are exposed to a closely related species, *D. persimlis*, a weak competitive response would be generated. However, if the males are exposed to the more distantly related *D. melanogaster*, there would be a similar competitive response to being raised in the absence of pseudorivals.

1.3 The Evolution of Hybrid Male Sterility

1.3.1 *The Evolutionary Genetics of Hybrid Male Sterility*

Sperm competition has been identified by several studies as a potent force driving rapid evolution (6, 63–65). It is not surprising, therefore, that theoretical models predict sexual selection acting on allopatric populations could result in rapid genetic divergence between those populations (4, 66–68). Should the populations continue along divergent evolutionary paths, they may eventually develop reproductive isolation. There exist two types of reproductive barriers maintaining biodiversity, both of which are commonly

found (69). The first is prezygotic isolation, wherein two species do not mate to produce offspring. This is often due to one species not recognizing the other as a potential or optimal mate. The second mechanism maintaining biodiversity is a barrier that exists following the formation of the zygote (postzygotic isolation); this includes hybrid sterility and inviability.

The prevalence of postzygotic reproductive barriers throughout the animal and plant kingdoms caused scientists to focus on the role it plays in speciation. Charles Darwin first noted the prevalence of hybrid sterility and inviability amongst interspecies hybrids (70). However, it was a great mystery to Darwin how such a deleterious phenotype could evolve by natural or sexual selection. This conundrum wasn't resolved until Bateson, Dobzhansky and Muller independently formulated the same theory on how hybrid dysfunction could evolve, a theory known today as the Bateson-Dobzhansky-Muller (henceforth BDM) model (71, 72).

Under this model, divergence at two loci is required in order to develop hybrid incompatibilities. Consider an initial population that is split into two geographically-isolated subpopulations (Figure 1.1). Over time, the two subpopulations could undergo divergent adaptive evolution at separate, yet interacting, loci. Since the newly-arisen novel alleles are adaptive, they may become fixed within each respective population. Should the geographic barriers separating the subpopulations break down, either through the physical breakdown of the geographic barrier, or if the populations are brought together in a laboratory setting, the hybrids formed between these two populations will receive one ancestral and one derived allele at both loci. The derived traits may be incapable of proper epistasis as they have never been present in a common genetic

background, and therefore have never undergone natural or sexual selection in combination with one another. As such, the combination of these two derived alleles could result in a dysfunctional interaction, such as hybrid sterility or inviability (Figure 1.1).

The genetic basis of postzygotic isolating barriers was further advanced when it was determined that hybrid sterility and inviability predominantly affects the heterogametic sex (XY or ZW). This trend, called Haldane's rule, has been identified as being true for nearly all species identified to date in *Drosophila* (flies), birds, mammals, *Aedes* (a genus of mosquitos), *Lepidoptera* (butterflies and moths), and *Anopheles* (a genus of mosquitos)(73–78). Muller was the first to connect the BDM model to Haldane's rule (79). He suggested that the combination of a recessive X-linked locus epistatically interacting with an autosomal dominant locus would produce the sterile or inviable phenotype preferentially in heterogametic hybrids (Figure 1.2). Such a theoretical framework sets the stage for the identification of speciation genes(80–82). In the initial hunt for speciation genes, much focus was given to hybrid inviability (83–87). This is partially because hybrid inviability is a striking feature that seemed to be critically important in isolating species, and also because it is a relatively easy phenotype to score. As a result of this focus, several genes have been identified that contribute to hybrid inviability in many different species pairs (88). It was later shown through mathematical modelling, as well as with comparative evidence from species in *Drosophila*, birds, and fish, that hybrid inviability may not be an important postzygotic barrier during the initial stages of speciation since it has been shown to evolve at a slower

rate than hybrid male sterility (73–78). As a result, it is believed that hybrid male sterility

is more important in the initial stages of speciation.

There are several evolutionary mechanisms that are believed to result in F_1 hybrid

sterility in allopatric animal populations. One rarely-discussed evolutionary path is sperm

competition (68, 89, 90). As previously mentioned (see subsection 1.1.1), sperm

competition is a potent driver of the evolution of reproductive characteristics, and traits

related to sperm production are some of the most rapidly-evolving (64, 91–95). Should

two populations become geographically isolated, it is unlikely that sperm competition

would act similarly in both instances. As a result, sperm competition will result in

divergent evolution of reproductive characteristics (4). If the sperm competition between

the two species acted upon the gametes, then it is likely there would be divergent

evolution for the developmental processes underlying gamete production. Divergence for

such traits taking place at epistatically interacting loci could, upon secondary contact and

hybridization, result in hybrid sterility according to the Bateson-Dobzhansky-Muller

model. Furthermore, since sexual selection can drive rapid evolution, it is expected that

such a path to reproductive isolation could rapidly take place.

1.3.2 *Meiotic Drive and Hybrid Sterility*

One well-discussed evolutionary process leading to hybrid sterility is genetic conflict (88,

96). Selfish genetic elements function to prevent transmission of alleles that do not

contain the selfish elements. Meiotic drive elements function either during or after
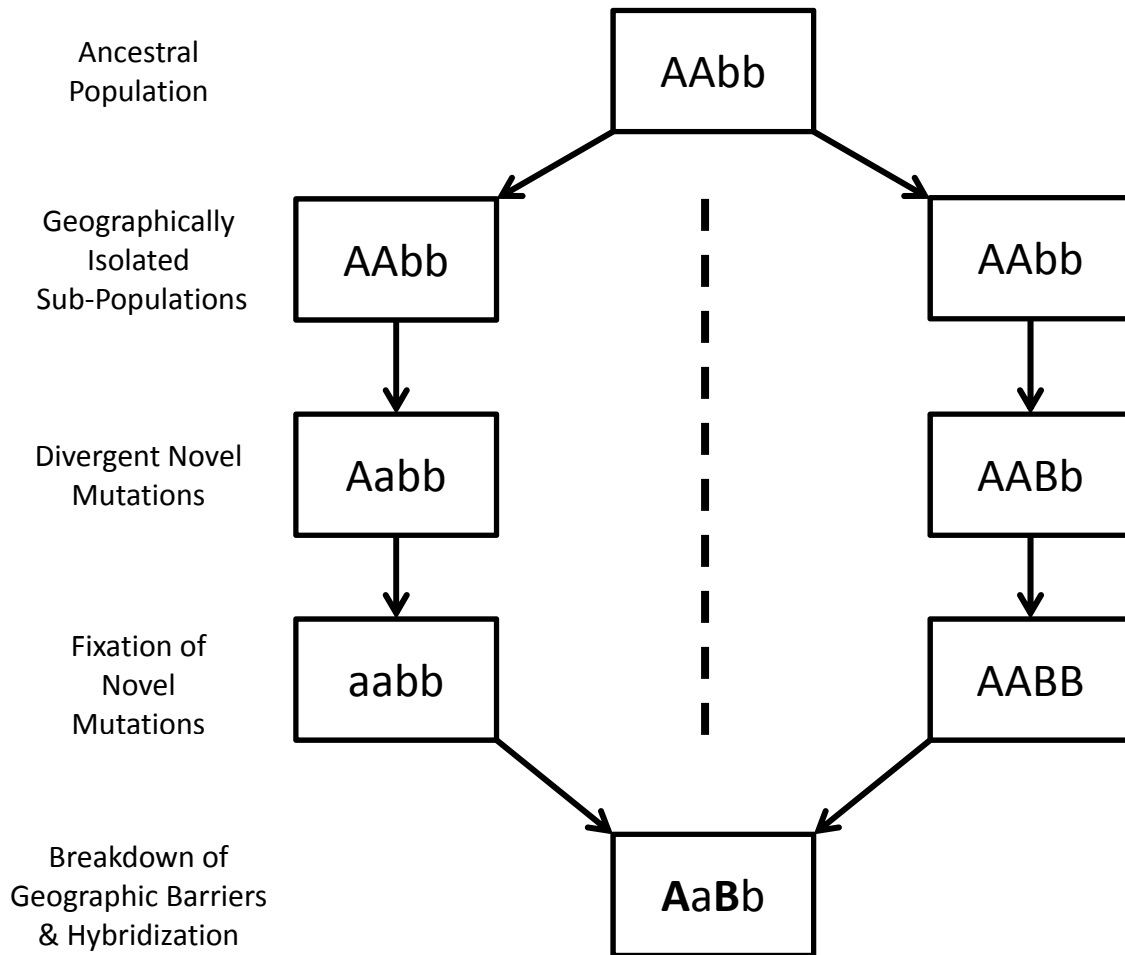
Figure 1.1 The Evolution of Hybrid Incompatibilities through the Bateson-Dobzhansky-Muller Model. An initial population of individuals (genotype AAbb where locus 'A' and 'B' interact) is split into two geographically isolated subpopulations. One population may undergo divergence at the 'A' locus, while the other population diverges at the 'B' locus. Should these novel mutations be beneficial, they can become fixed in the separate populations. When the geographic barrier breaks down, the populations may be able to hybridize. Hybrid individuals will have genotype of AaBb. The alleles of 'a' and 'B' have never been together in a common genetic background, and thus, never faced natural selection. This could lead to hybrid incompatibilities should the interaction of the 'a' and 'B' alleles prove deleterious.

A
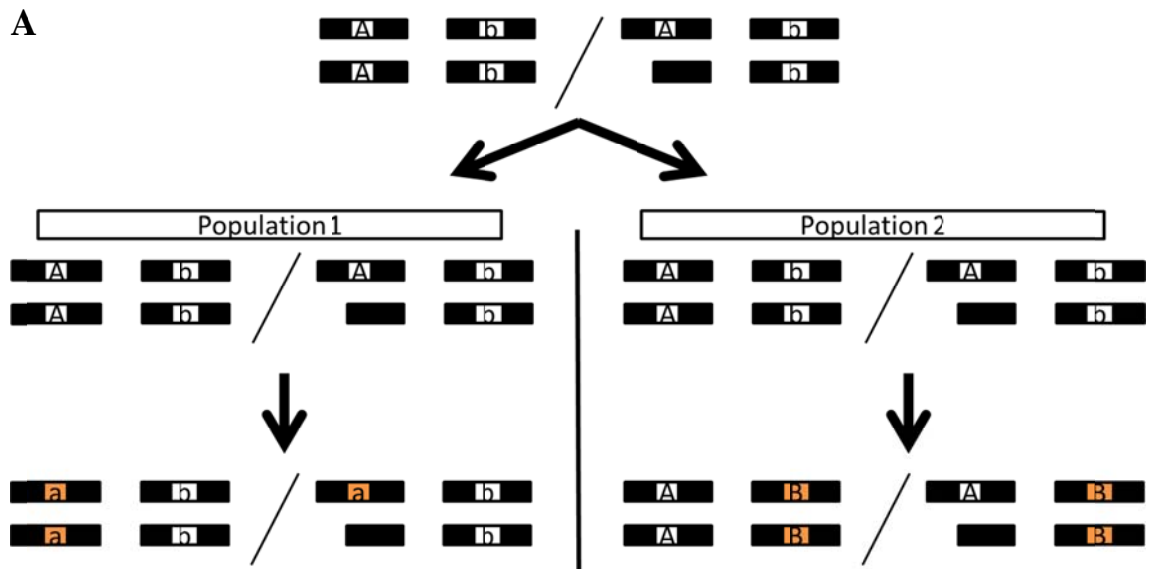
B
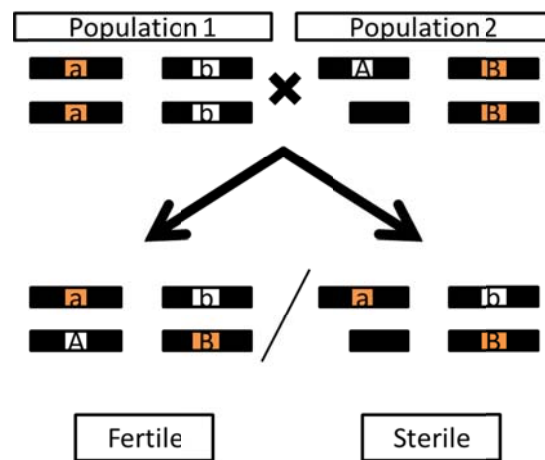
Figure 1.2 Evolution according to the Bateson-Dobzhansky-Muller model can explain preferential sterility of the heterogametic sex (Haldane's Rule). Horizontal black bars represent chromosomes. Chromosomes harbouring the 'A' locus are sex chromosomes, while chromosome without a letter represent heterogametic chromosome (i.e. Y or W). Chromosomes harbouring the 'B' locus are autosomes. An initial population comprised of homogametic (i.e. XX) and heterogametic (i.e. XY) individuals is split into two geographically isolated subpopulations (A). Population 1 undergoes divergent evolution at the 'A' locus, fixing the recessive 'a' allele (A). Population 2 undergoes divergent evolution at the 'B' locus, fixing the dominant 'B' allele (A). Upon breakdown of the geographic barrier, the two populations come into contact with the opportunity to mate (B). Homogametic individuals from Population 1 can mate with heterogametic individuals from Population 2 (B). Resulting homogametic offspring have interacting chromosomes that have previously been exposed to natural selection and are, therefore, fertile. Heterogametic individuals have alleles that have not previously been exposed to natural selection unmasked ('a' interacting with 'B') potentially producing sterile interactions.

One well-discussed evolutionary process leading to hybrid sterility is genetic conflict (88, 96). Selfish genetic elements function to prevent transmission of alleles that do not contain the selfish elements. Meiotic drive elements function either during or after meiosis to prevent transmission of the corresponding non-selfish allele into gametes (97). This produces segregation distortion whereby one allele is underrepresented in the next generation. If the meiotic drive element lies on a sex chromosome, the segregation distortion will produce altered sex ratios. Segregation distortion reduces male fitness by reducing the number of sperm capable of fertilization. As a result, there is strong drive for the suppressors of segregation distortion (98). Since both the induction and suppression of the drive system is expected to evolve rapidly, a particular meiotic driver and its corresponding suppressor are not expected to be identical between geographically isolated populations (99). Therefore, when hybrids between two populations are made, the suppressor or the driver may be unleashed (96, 100, 101).

A meiotic drive system has been implicated for the only hybrid sterility gene identified in mammals. *Positive regulatory domain zinc finger protein 9* (*Prdm9*) appears to have evolved as an autosomal drive suppressor that produces sterility in hybrids formed between *Mus musculus musculus* females and *M. m. domesticus* males (102). *Prdm9* functions during meiosis I where it localizes recombinatorial machinery to the proper loci (103, 104). It also appears to function during meiotic sex chromosome inactivation (MSCI), a seemingly mammalian-specific feature of spermatogenesis where there is silencing of nearly all X- and Y-linked genes shortly after the initiation of meiosis. Meiotic drive theory predicts that MSCI is a likely suppressor to sex ratio distorters present on the X and Y chromosomes that may be functioning during meiosis

(103). Since suppressors of meiotic drive co-evolve with the driver element, it is predicted that separate lineages would potentially evolve separate MSCI mechanisms. When combined in a common genetic background, this could produce disrupted MSCI, resulting in disrupted meiosis. Interestingly, the F_1 hybrid males produced from a *M. m. domesticus* mother are fertile and show normal MSCI, while F_1 males from a *M. m. musculus* mother are sterile and show disrupted MSCI (105). These results strongly implicate the MSCI function of *Prdm9* in hybrid sterility and suggest it may have evolved as a suppressor of meiotic drive.

A second meiotic drive system that has diverged between *Drosophila simulans* and *D. mauritiana* has also been identified. Between these species, a region of the *D. mauritiana* third chromosome produces segregation distortion when present in a *D. simulans* genetic background (106, 107). The corresponding suppressor element between these species has not yet been identified. When the genomic region containing the drive element was introgressed from one species into the other, some of the resulting introgression lines with the drive element also showed reduced fertility. This implies that the region contributing to hybrid male sterility between these lines could potentially result from the evolution of the meiotic drive system.

A third meiotic drive system that has a major role in hybrid sterility is found between two recently diverged subspecies of *D. pseudoobscura*. One of the subspecies' range spans from British Colombia to Guatemala (*D. p. pseudoobscura*; henceforth USA)(108). The second subspecies is an allopatrically isolated group found exclusively in the area directly surrounding Bogota, Columbia (*D. p. bogotana*; henceforth BOG)(108). These species diverged only 150,000 years ago, presenting a rare

opportunity to study very recently diverged subspecies (109–111). Furthermore, hybrid

sterility between the subspecies is incomplete, only manifesting in F_1 males produced

from BOG mothers (henceforth F_1^{BOG}), providing further evidence of their recent

divergence (112). In a novel study, it was identified that the defects leading to sterility

become less severe in F_1^{BOG} males as they age, resulting in weakly fertile males (113).

This is the first ever reported case of hybrid sterility becoming less severe as the hybrids

are allowed to age two weeks. These aged fertile males produce almost exclusively

female offspring, suggesting a close link between hybrid sterility and meiotic drive in this

pair of subspecies.

It was later identified that a single gene present on the X chromosome was

necessary, but not sufficient, to produce both the hybrid sterility and meiotic drive

phenotypes (100). Due to its role in producing an overabundance of females, this gene

was called *overdrive* (*ovd*). It appears *ovd* epistatically interacts with several genomic

regions to produce the sterility and meiotic drive phenotypes (100, 101). Several of these

interacting regions influence both hybrid sterility and segregation distortion, further

suggesting hybrid sterility and segregation distortion may have a single causal link.

1.3.3 *Spermatogenesis in* **Drosophila**

Although the evolutionary genetics of hybrid sterility genes have been well documented

across several taxa, how they actually produce the sterility phenotype is largely ignored.

It is suggested that spermatogenesis is disrupted in sterile F_1 hybrid males, but it has not

been determined when fertility is disrupted during spermatogenesis (69). In *Drosophila*,

spermatogenesis is a cellular process where small diploid cells with limited motility are

converted to long, mobile string-like haploid cells (Figure 1.3)(114). The stages that take place during spermatogenesis have been well-documented for *D. melanogaster*, and a limited assessment of spermatogenesis in related species has shown that the process is similar across *Drosophila* species. In *D. pseudoobscura*, spermatogenesis takes place entirely within two ellipsoidal testes connected via a lateral seminal vesicle. Spermatogenesis progresses from the apical tip of the testes towards the distal end (116). Within the apical tip, a somatic hub is surrounded by several germline progenitor stem cells, each of which is surrounded by two cyst progenitor stem cells (117, 118). These are the stem cells that give rise to all spermatogenic products, and ultimately, all sperm the male will produce.

Spermatogenesis begins with a self-renewal mitotic division of the germline stem cells (114). The plane of division moves away from the somatic hub. When the cells separate following the completion of mitosis, one cell maintains a physical association with the somatic hub, while the other cell progresses distally. The cell which maintains physical association will repeat the above self-renewal division, a process mediated by the JAK-STAT signalling pathway through Unpaired secretion by the somatic hub (117, 118). This renewal process ensures the maintenance of the stem cell population within the testes, allowing for continual production of sperm throughout the life of the fly. The cell which moves distally away from the hub does not have the JAK-STAT signalling pathway activated. As a result, it does not return to a stem cell state and becomes a primary spermatogonium. Each pair of cyst progenitor cells also undergo a self-renewal division (again mediated by the JAK-STAT signalling pathway), producing a cyst progenitor cell as well as a cyst cell which
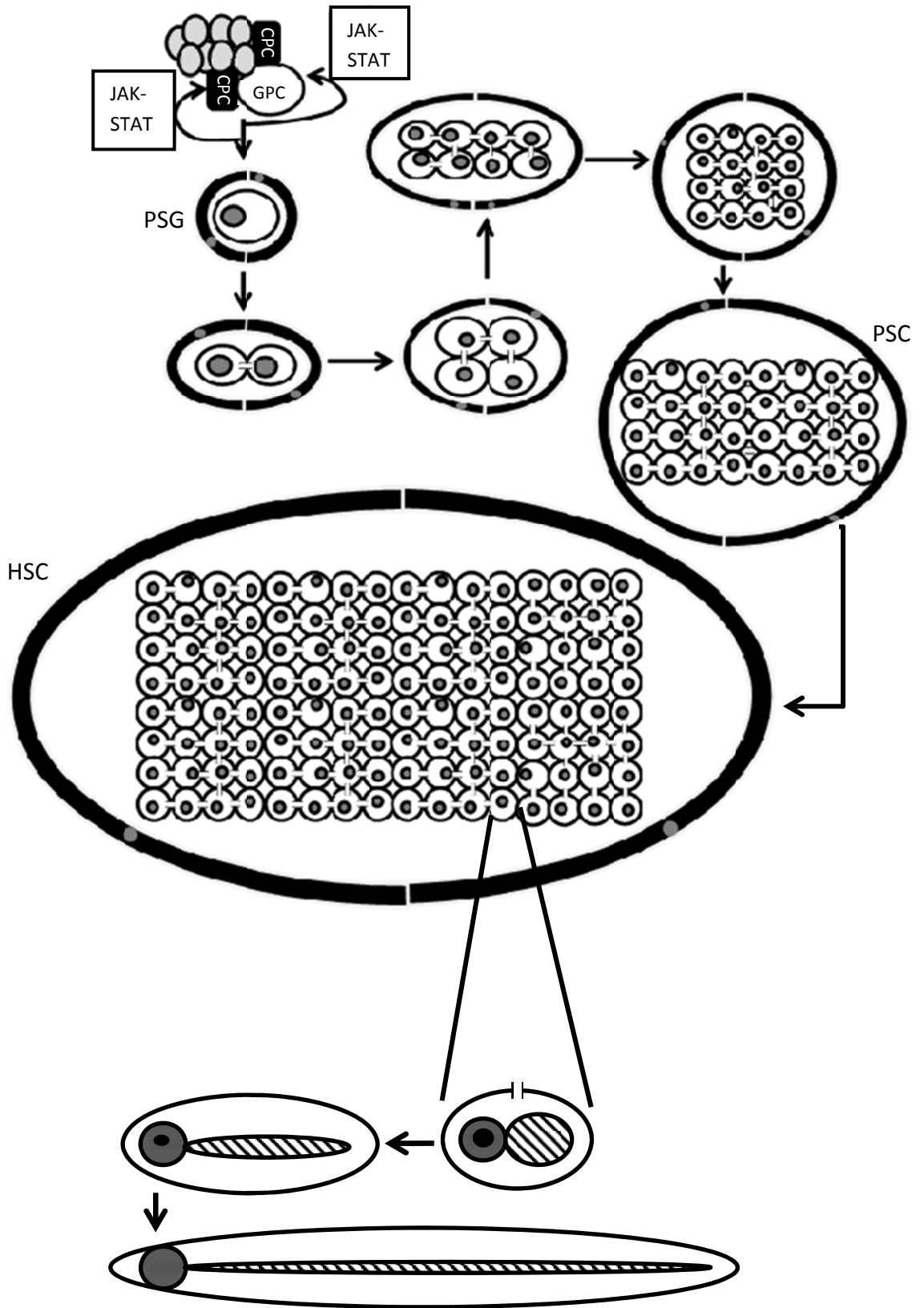
Figure 1.3 Spermatogensis in Drosophila pseudoobscura. At the apical tip of the testes, a bundle of 8-9 somatic cells (light grey ovals) physically associate with cyst progenitor cells (which give rise to cyst cells; CPC) and germline progenitor cells (which give rise to resulting sperm cells; GPC). A mitotic division of the cyst progenitor cells and germline progenitor cells displaces a cyst cells (black semi-circles) and primary spermatogonia (white ovals with grey nuclei within) away from the hub. The resulting daughter cell from the mitotic division that does not displace away from the hub is converted back to progenitor cells via the JAK-STAT cascade. The primary spermatogonia undergo five rounds of mitotic divisions while the cyst cells do not divide, but rather grow to accommodate the growing volume. This produces a 'bundle' of 32 primary spermatocytes (PSC) which undergo large-scale cellular growth. When growth is complete, meiosis takes place, producing 128 haploid spermatocytes (HSC). Following meiosis, all the mitochondria within each cell aggregate to produce the nebenkern (white circles with diagonal black lines). During this stage, a dark nucleolus forms within the nucleus (dark black circle). As the nebenkern begins to elongate, the nucleolus shrinks and then fades. As elongation of the nebenkern progresses, nuclear elongation commences, producing the characteristic string-like sperm shape of *Drosophila* sperm. Image not to scale. Modified from Kanippayoor, Alpern and Moehring (115).

encapsulates the primary spermatogonium. The cyst cells surrounding the primary spermatogonia will not divide again and will continue to surround all of the cells resulting from the division of a single primary spermatogonium. When the primary spermatogonium has proceeded through spermatogenesis, the cyst cells release the developed sperm.

Following a round of mitosis, two cells called secondary spermatogonia are present within the two cyst cells (114). The secondary spermatogonia will undergo several rounds of mitosis to produce primary spermatocytes, the cells that will eventually undergo meiosis. The number of mitotic divisions of the primary spermatogonia, and subsequently the number of primary spermatocytes that are within each pair of cyst cells, varies among the species of *Drosophila*. It ranges from three divisions (eight primary spermatocytes) as in *D. virilis* to as many as six divisions (64 primary spermatocytes) in *D. coracina* (*D. melanogaster* undergoes four divisions producing 16 primary spermatocytes; *D. pseudoobscura* undergoes five divisions producing 32 primary spermatocytes)(119). In all species of *Drosophila* tested to date, the primary spermatocyte stage is an extended growth phase (120). Each cell grows drastically in volume (roughly 25-fold, although this increase is likely variable) with high expression levels of genes associated with male fertility. After the extensive growth and expression phase is completed, the primary spermatocytes undergo meiosis, producing haploid postmeiotic spermatids (128 in *D. pseudoobscura*).

Most gene products that are required postmeiotically are transcribed during the primary spermatocyte stage and stored until needed following meiosis (121). However, there is evidence of expression of genes on the X chromosome following meiosis (122,

123). Since secondary spermatocytes are haploid, not all of them contain an X chromosome. Y-bearing spermatocytes would therefore not have the X chromosome products that are postmeiotically expressed. To get around this problem, all cytokinetic events that take place during spermatogenesis, either during mitosis or meiosis, are incomplete (124, 125). This incomplete cell division leaves small cytoplasmic bridges connecting all spermatocytes. These bridges likely function to allow either passive or active transport of mRNA produced following meiosis.

Once meiosis is complete, all of the mitochondria within each spermatocyte cell form two aggregates (a major and a minor mitochondrial aggregate) which physically associate as one large mass called the nebenkern (126). During this aggregation, the mitochondria cristae reform and fuse, producing a layered appearance in the nebenkern, leading to this stage of spermatogenesis being colloquially named the "onion stage." During the onion stage, the axoneme, a cytoskeletal structure that makes up the sperm flagellum, extends from the basal bodies on one side of the nucleus (114). The axoneme is composed of microtubules that are aligned in parallel and functions as a scaffold along which the major and minor mitochondrial aggregates elongate to the appropriate length, ultimately forming the sperm tail. The sperm head in *Drosophila* closely resemble the sperm tail, as they also elongate, achieving a width identical to that of the sperm tail(127). During the elongation of the sperm nucleus, the cytoplasmic bridges are closed off, producing individualized sperm. The elongating sperm coil, and eventually break free from the cyst cells in which they are contained. These mature sperm progress into the seminal vesicle where they are stored until ejaculation.

In Chapter Four I evaluate the phenotypic manifestations during spermatogenesis of hybrid male sterility and meiotic drive. Previous reports have shown an overlap between meiotic drive and hybrid sterility across several taxa (100, 101, 103, 106, 107). As a result, it is predicted that there is a similar genotypic and phenotypic basis of male sterility and unleashed meiotic drive systems in hybrids. To test this, I looked to assess the mechanisms that lead to hybrid sterility between *D. pseudoobscura pseudoobscura* (USA) and *D. p. bogotana* (BOG). I then looked at the residual spermatogenic errors once the initially sterile hybrids became fertile but produce sex-ratio-biased offspring, suggesting which spermatogenic phenotypes contribute specifically to segregation distortion. I hypothesized that if certain spermatogenic errors were alleviated once initially sterile males became fertile, then the alleviated spermatogenic errors are causing hybrid male sterility, while the residual spermatogenic errors are producing segregation distortion.

A previous experiment identified that, in hybrids between USA and BOG, when a small region of the BOG X chromosome was replaced with USA loci through introgression, both hybrid sterility and segregation distortion phenotypes were alleviated (100). The authors were able to map the phenotype down to a single gene, *ovd*. As a result of these experiments, I wanted to determine the manner in which the speciation gene *ovd* contributes to hybrid sterility and meiotic drive by replicating their introgression experiments and characterizing spermatogenesis in these introgression hybrids. I hypothesized that if *ovd* contributes to both hybrid male sterility and meiotic drive, then introgressing the USA *ovd* allele should eliminate the hybrid sterility and meiotic drive spermatogenic phenotypes observed in F_1 hybrids.

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Chapter 2

Identification of a novel sperm class and its role in fertilization in *Drosophila*

2.1 Introduction

Following copulation, male sperm experience various selective pressures on the path to fertilize an egg, such as inherent selective pressures produced by the female reproductive tract (1, 2) and inter-male sperm competition (3, 4). To combat these obstacles and increase fertilization success, males of many species will produce two distinct types of sperm: non-fertilizing parasperm and fertilizing eusperm (reviewed in ref. 5). Although parasperm are not directly involved in fertilization, they often represent a large proportion of ejaculate content (6-9), suggesting a maintained and important function in fertility throughout vertebrate and invertebrate taxa. As such, parasperm are primarily thought to function in sperm competition (10, 11) and eusperm protection (9, 12-14), the latter of which has some experimental support (15). While as many as four types of parasperm can be produced by a single male, depending on the species, only one of these varieties of parasperm have been shown to be deposited into the female reproductive tract during a copulation event (5). It is possible that, instead, multiple morphologically similar parasperm types are transferred during copulation, each potentially with a subfunctionalized role in male fertilization success. However, the

indistinguishable nature of the parasperm prevents the ability to experimentally address whether role subfunctionalization is occurring.

The most widely studied insect genus, *Drosophila*, provides only one subgroup of species which possess parasperm, the *obscura* subgroup (16). Within this group, the best-studied species is *Drosophila pseudoobscura*, in which males have been reported to produce a single type of parasperm (17, 18). The parasperm and eusperm in this species are differentiated based on their length, with the parasperm being approximately $1/5^{th}$ the length of eusperm. The parasperm within this group has previously been reported as having a bimodal size distribution (15), suggesting that the single class of parasperm may actually be comprised of two morphs with overlapping sizes. Using a strain of this species, we report the first documented case of two distinct and separate morphological classes of parasperm being transferred into the female reproductive tract, and further show that these two parasperm types have subfunctionalized roles in fertilization.

2.2 Materials & Methods

2.2.1 Drosophila *maintenance*

All flies used in this experiment were *Drosophila pseudoobscura pseudoobscura* (line 149; 14011-0121.149 from the *Drosophila* Species Stock Center, San Diego, CA) reared on a standard yeast-cornmeal-agar medium (Bloomington Drosophila Stock Center) and maintained and tested at 22°C.

2.2.2 *Sperm morphology measurements*

Sperm length was determined by measuring sperm transferred to females to ensure the values were biologically relevant and all sperm measured were fully developed. Five-day-old virgin males were individually paired with a virgin female and allowed to mate. Upon completing copulation, the male was discarded and the female was left for a period of 30 minutes to allow sperm to travel up from the bursa copulatrix. The female's reproductive tract was then dissected in phosphate buffered saline (PBS) on a slide to release the transferred sperm. The solution was mixed by gently pipetting up and down to ensure minimal clumping and even dispersal of sperm. A drop of 5 µl of the sperm solution was placed on a microscope slide and visualized at 100X magnification under phase contrast microscopy using a Nikon Eclipse E1000 microscope. To measure nuclear length, a 1µl drop of 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) was added to the sperm solution on the slide, left for 5 minutes, then visualized using a Leica DMI6000 B Inverted Microscope. All lengths were calculated using Nikon NIS-Elements Software. For visualization of the associated string-like structure (Figure 2), a 5µl aliquot was transferred to 45 µl of 45% acetic acid fixative and also visualized using a Nikon Eclipse E1000 microscope.

2.2.3   *Parasperm and competition*

To test if one of the parasperm morphs were responding to sperm competition, males were placed in either an isolated condition (one male in one vial) or a competitive condition (10 males in one vial) within 2 minutes of eclosion. Both social conditions (isolated and competitive) were allowed to age for 5 days, after which the males were tested in a competitive (two males, one female) or isolated mating assay (one male, one

female). This ultimately produced four different treatment conditions: Isolated

Social/Isolated Mating, Isolated Social/Competitive Mating, Competitive Social/Isolated

Mating, and Competitive Social/Competitive Mating. Immediately upon completion of

copulation, all males were removed from the vial to ensure only the mated male's

ejaculate would be present for quantification. The female was left in the vial for a period

of 30 minutes. The mated female's reproductive tract was then dissected as above. All

sperm from a 5 µl diluted sample (1:100) were counted using a Nikon Eclipse E1000

microscope and classified as parasperm 1, parasperm 2, or eusperm based on length

parameters as determined above.

2.2.4   Parasperm and female spermicides

A previous report indicated that parasperm in *D. pseudoobscura* functions as a

protection from female spermicide (15). To test whether the two parasperm classes both

provide this protection, we replicated the *in vitro* methods indicated in that study. In

brief, we dissected sperm from the male's reproductive tract and added 1.25µl of mature

sperm collected from a single male's reproductive tract to 1.25µl of female reproductive

tissue, female muscle tissue from the thorax, or saline. The samples were then left for 25

minutes, at which time a Live/Dead stain (11 µl of SYBR®-14 and 22 µl of propidium

iodide per 0.5 ml PBS) was applied; SYBR®-14 stains live cells green and propidium

iodide stains dead cells red. The number of live and dead eusperm was quantified using a

Leica DMI6000 B Inverted Microscope. All methods were identical to (15), except that

we extracted 20 samples of reproductive tract or muscle into 20µl (rather than 100 into

100µl) four separate times, and we used a siliconized plastic pestle (rather than glass) for homogenizing the samples.

2.2.5 *Statistics*

All statistical tests were carried out either in R Statistical Software version 2.15.2 (Vienna, Austria) or Microsoft Excel 2010.

2.3 Results & Discussion

Using light and fluorescent microscopy, and taking particular care in sample preparation to not damage or break the sperm, we first measured the length of sperm a male deposits into the female reproductive tract. The length measurements fit into three discrete categories that are significantly different from one another (Figure 2.1A,B; Tukey's Post Hoc, *P*<0.0001 for all). Previous reports identified that only the longest sperm class was fertilizationally competent in *D. pseudoobscura*, a report that has since been verified to be the case 100% of the time (17, 18). As such, we assume that both the medium and short sperm are functioning as parasperm: parasperm 1 (55.36±2.34 µm) is approximately half the length of parasperm 2 (101.11±2.38 µm). The eusperm length measured in our study (302.15±9.62 µm) corresponds to that previously reported for this sperm class, while parasperm 1 and 2 overlap the range previously reported for what was considered a single parasperm class in this species (15).

To verify that the presence of two classes of parasperm is not simply due to damaged sperm resulting from gross errors during spermatogenesis, we observed the motility of the sperm across a range of biologically-relevant situations. We found that all

three sperm classes are motile when extracted from fertile males (100%, n=20), are motile within the female's reproductive tract within two hours of mating (100%, n=20), are initially stored in the female's long-term sperm storage organs (spermathecae) two hours after mating (100%, n=20), and have persisted in these organs five days after mating (100%, n=10). We then wanted to determine if the two classes of parasperm are physically distinct from one another beyond their length differences. Commonly, parasperm have shown to increase in length with the increase in the size of the nucleus.

We found a strong correlation between head and tail length when comparing across sperm types (r=0.979; Figure 2.1C), but a moderate to weak correlation within each sperm type (parasperm 1: r = 0.386, n=24; parasperm 2: r = 0.304, n=19; eusperm: r = −0.232, n=19), indicating that longer sperm types have longer nuclei, but variation in head and tail length within each sperm type do not necessarily coincide. Interestingly, we also noted that every observed parasperm 2 has a distinct spiral or wave conformation along the length of the tail, and eusperm have a slight waved conformation, while parasperm 1 do not have either of these features (Figure 2.1A). Upon treatment with a fixative, a previously-unreported string-like structure dissociates from both parasperm 2 and the eusperm (Figure 2.2), and the spiral or wave conformation is no longer present; both the wave and the string-like structure are not present with parasperm 1 (Figure 2.1A, Figure 2.2). This string-like structure does not appear to contain chromosomal material when treated with a nuclear stain (data not shown). Since the spiral or wave shape and string structure are not present with parasperm 1, and dissociation of the string-like structure appears to remove the spiral shape from parasperm 2, it appears that it may be involved in the formation or maintenance of bends in the sperm tail.
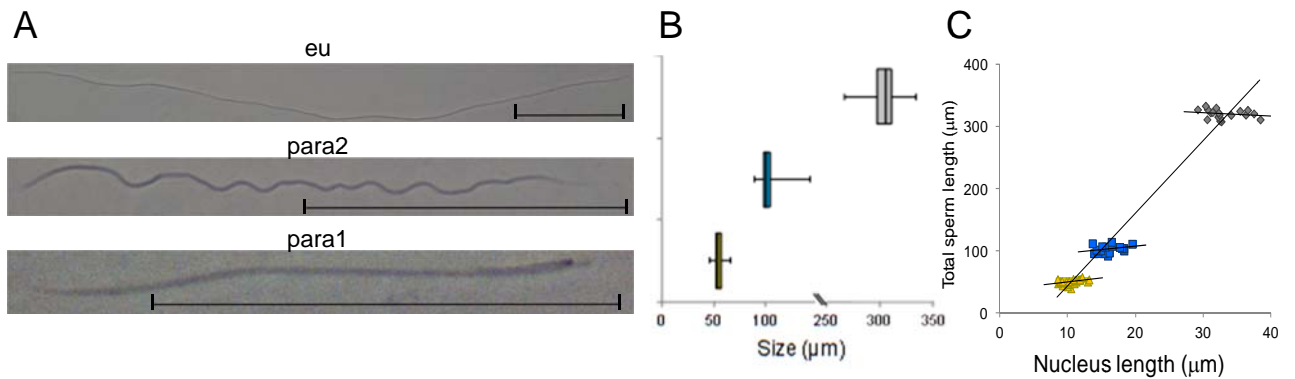
Figure 2.1 *Characterization of two parasperm morphs.* (A) Representative images of eusperm (eu), parasperm 2 (para2), and parasperm 1 (para1; 40x magnification; scale bar is 50μm for each image). Note the increased curvature of parasperm 2 compared to parasperm 1. (B) Box plots corresponding to the aligned images in (A) for total sperm length (N_{eu}=114, N_{para1}=141, N_{para2}=170); boxes are the upper and lower quartiles with the horizontal line representing the median and the vertical bars representing the maximum and minimum values recorded. Note that none of the sperm sizes overlap for the three groups. (C) Correlation of sperm head with total sperm length for parasperm 1 (yellow triangles), parasperm 2 (blue squares) and eusperm (grey diamonds).
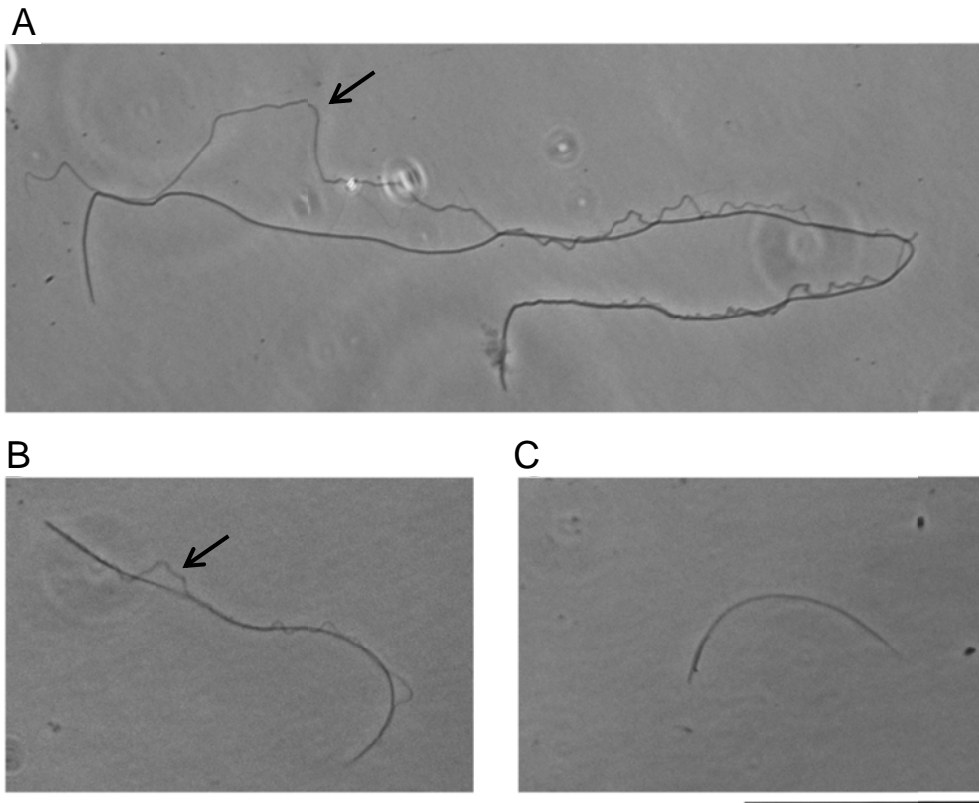
Figure 2.2 *Sperm-associated string structure.* The sperm-associated string structure (arrow) wrapped around both the (A) eusperm and (B) parasperm 2, but not found in (C) parasperm 1. Images are fixed in 45% acetic acid and imaged under phase contrast microscopy (40x magnification). Scale bar is 50μm.

We then tested whether males transfer different quantities of the two parasperm classes to the female during copulation. The two parasperm classes are differentially transferred to the female reproductive tract: 21% parasperm 1, 26% parasperm 2, and 52% eusperm (average 578 transferred sperm counted per sample, n=20 at 2 hours post-mating). When grouped together, the proportion of parasperm (1 and 2) to eusperm transferred to the female closely resemble the proportion reported in previous studies (15, 19). There also appears to be a trade-off between parasperm production and eusperm production; when counting the number of sperm transferred to a female there is a negative correlation between the amount of eusperm transferred and the amount of parasperm 1 ($r = -0.708$, $P<0.0005$) and parasperm 2 ($r = -0.65506$, $P=0.0017$) that is transferred.

Since the two parasperm morphs are differentially transferred to the female, our next goal was to identify if parasperm 1 and 2 were functionally different from one another. We wanted to test whether the two parasperm types have distinct functions, the most likely (and testable) of which would be acting in sperm competition and protecting eusperm from female spermicides. The ability to adjust ejaculate composition on the bases of competition in the attempt to maximize fitness has been observed in many organisms including insects (20-26), mammals (27, 28), and fish (29-31). Thus, ejaculate adjustment may be a common form of increasing competitive capabilities in males. It is, however, unclear how males are acknowledging the presence of other males. Some studies suggest that males are recognizing the male through pheromonal cues from competing males (25, 32), although evidence is mounting that numerous cues can be used simultaneously to determine the optimal ejaculate output (21, 33-35), which is likely the

case in *D. pseudoobscura* (36). To test whether parasperm play a role in sperm competition, and if this role is subfunctionalized within one parasperm class, we assessed males placed in different social environments and scored whether they compensated for the potential presence of sperm competition by adjusting the ratio of sperm types they deposited within the female reproductive tract. Males were collected immediately upon eclosion and placed either in isolation or in a potentially competitive group of 10 males for five days to establish their 'developmental' social condition, during which males could potentially adjust sperm type ratios during spermatogenesis. We then subdivided these two groups of males and assayed them in one of two different 'current mating' social conditions, during which males may potentially adjust the amount of each sperm type they transfer to the female: we placed one female in a mating assay with a single male (e.g., isolated), or placed one female with two males of the same treatment group (e.g, competitive). This created four treatment groups that vary in the male's initial developmental and current mating social experiences (n=20 per group). Thirty minutes after the completion of copulation, females were dissected and the ratio of sperm types was scored based on sperm length.

As the environment becomes more potentially competitive, males increase the amount of parasperm 2, and decrease the amount of parasperm 1 and eusperm in the ejaculate (Figure 2.3). The proportion of parasperm 2 is affected by both the initial developmental (dev) and current mating (mat) social context ($F_{1,76}$=59.47, P_{dev}<0.0001, $F_{1,76}$=5.98, P_{mat}=0.017, $F_{1,76}$=0.26, $P_{dev*mat}$=0.612; two factor ANOVA). These results indicate that males adjust the amount of parasperm 2 in response to the presence of competing males, regardless of whether those males are present well before mating or
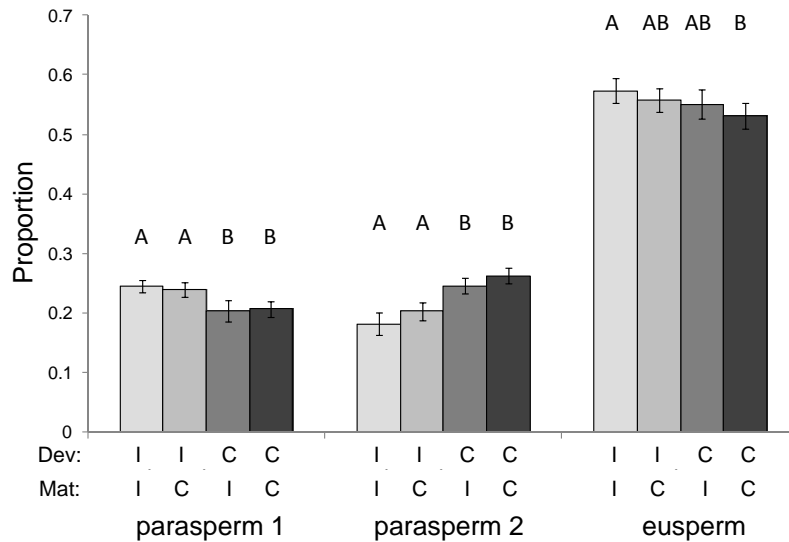
Figure 2.3 *Proportion of parasperm and eusperm in ejaculate across social conditions.* The proportion of each sperm type present in the female's reproductive tract was measured for males experiencing different combinations of social condition [isolated (I), housed singly *vs.* competitive (C), with other males] at two time points [during the first five days after eclosion (developmental: Dev), and when paired with a virgin female at day five (current mating: Mat)]. Different letters indicate significant difference due to social condition at p<0.05 (Tukey's Posthoc Test) within each sperm type.

within the mating arena, suggesting that parasperm 2 likely plays a role in sperm competition. Furthermore, since males adjusted the amount of parasperm 2 in response to the current mating condition, we show that the ejaculate response can occur effectively instantaneously. In contrast the proportion of parasperm 1 was reduced with increasing sperm competition, demonstrating that they are unlikely to be directly involved in sperm competition, and this reduction only occurred based on the developmental social context ($F_{1,76}=28.24$, P_{dev} <0.0001; Figure 2.3), and not current mating social context ($F_{1,76}=0.02$, P_{mat} =0.889, $F_{1,76}=0.5$, $P_{dev*mat}$ =0.484).

There is an important additional observation. As previous studies within *D. pseudoobscura* did not take into account the presence of two distinct parasperm classes (15, 17, 18, 19), it is possible that many species deposit multiple parasperm types with distinct functions, but these parasperm are morphologically indistinguishable from one another. If we pool our results from parasperm 1 and parasperm 2, as would be the case if they were morphologically indistinct, we are still able to detect a marginally significant change in parasperm quantity in response to the presence of sperm competition in the developmental social context, but not the current mating social context ($F_{1,76}=$ 4.91, $P_{dev}=0.03$, $F_{1,76}=2.59$, $P_{mat}=0.12$, $F_{1,76}=0.03$, $P_{dev*mat}=0.86$). It is conceivable that had this experiment been replicated, there may be no difference in parasperm quantity in these conditions as in previous studies. Thus, it is possible that many non-significant results from studies on parasperm function are potentially incorrect, since pooling the data from multiple parasperm types could dilute the ability to detect the effect of a single parasperm type.

Since parasperm 2 are deposited in the female even when males are raised and tested in complete isolation from other males, these sperm must either be developmentally constrained to always be produced at some level, or they have a secondary function, such as protecting the eusperm from proteins present in the female reproductive tract. The presence of *D. pseudoobscura* parasperm was previously shown to prevent eusperm death from female spermicides (15), and we expanded upon these results to test whether both parasperm types are involved in this process. If so, then the protective effect of parasperm may simply be due to physical parasperm presence, rather than a particular interaction, while any subfunctionalization of parasperm roles would indicate a specific mechanism by which one parasperm type (but not the other) is protecting eusperm. To test this, we collected sperm from the vas deferens, scored it for the proportion of each sperm type and exposed it to spermicide collected from the female reproductive tract (or thorax muscle tissue or saline as controls), then measured eusperm survival (19). When examining the total amount of parasperm, we find that eusperm survival is significantly correlated with the proportion of parasperm in the presence of the female reproductive tract (r=0.610, *P*=0.0072; Figure 2.4) but is not significantly correlated in the presence of muscle tissue or saline, with similar correlation values to those previously reported (data not shown)(15). When we separate the effect of the parasperm by individual morph, parasperm 1 and parasperm 2 convey equal protection for eusperm survival (r=0.390, r=0.430, respectively; Figure 2.4), suggesting that neither parasperm morph is individually responsible for protecting the eusperm, but rather that the proportion of eusperm to parasperm is crucial for limiting eusperm death from female
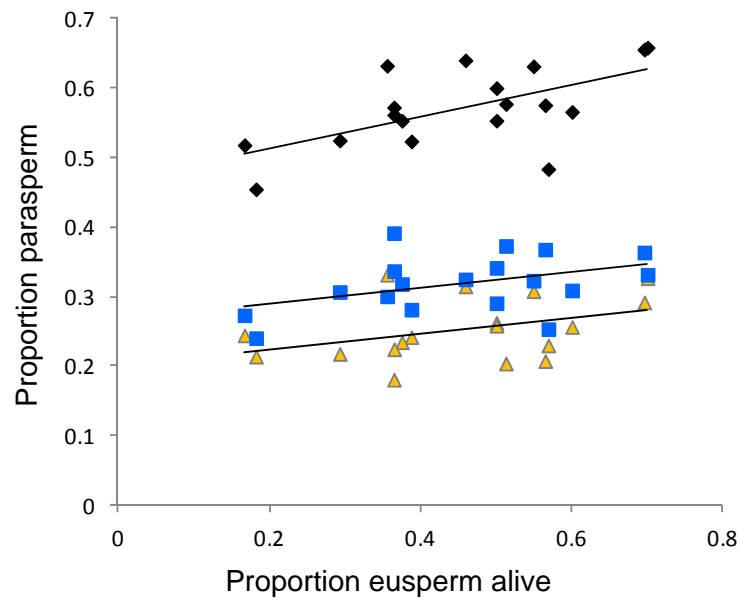
Figure 2.4 *Eusperm survival correlated with parasperm proportions.* The proportion of eusperm that is alive in the presence of female reproductive tract proteins is significantly correlated with the total proportion of parasperm (black diamonds; r=0.610, *P*=0.0072). Parasperm 1 (yellow triangles; r=0.390) and parasperm 2 (blue squares; r=0.430) contribute equally to eusperm survival.

spermicides. However, our results do not preclude the possibility of subfunctionalization of the parasperm towards different subsets of these spermicides.

The results from this chapter show the first evidence of multiple sperm morphs transferred in an ejaculate. Taking the sperm competition and spermicide results together, we propose that parasperm 2 has evolved to function in sperm competition. The longer length and corkscrew shape (Figure 2.1A,B) could, theoretically, aid in physically displacing competitor sperm. Our second proposition is that the presence of parasperm, any parasperm, protects eusperm from female spermicides. Since parasperm 1 is a shorter sperm morph, it potentially evolved as an energetically 'inexpensive' mechanism to increase protection from spermicide-related death. Thus, when males sense decreased levels of sperm competition, they respond by reducing energy spent on unnecessarily long parasperm, and instead compensate by increasing production of short parasperm.

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Chapter 3

Behavioural Plasticity in Response to Conspecific & Heterospecific Pseudorivals

3.1 Introduction

In many species, ejaculates from multiple males compete within the female's reproductive tract for a limited number of eggs (1). Males benefit from changes to traits that enhance their competitive capacity by allowing a higher paternity share (2). These changes have been well-documented both between species and within species, such as the correlation between the level of sperm competition and testes weight relative to body size (3–10). Species deemed more polyandrous put more energetic reserves into developing reproductive output to maximize fitness, whereas less polyandrous species put energy into somatic tissue. Within species, there can be large standing variation for certain ejaculate characteristics such as sperm length (6, 11–16). In these studies, reproductive traits strongly correlate with competitive fertilization success.

Some traits that are beneficial under instances of sperm competition are costly to produce and may be detrimental under reduced competition (17, 18). As such, it is predicted that, if levels of sperm competition vary, males would evolve phenotypic plasticity for traits that are beneficial only under high (or low) competition (19, 20). One way to assess the level of plasticity for these traits has been to induce perceived risk or

intensity of sperm competition for a focal male and observe changes in the ejaculate that impact competitive capacity (21–29). A variety of ejaculate characteristics have been shown to respond to an increase in the potential for sperm competition, such an increase in the amount of sperm, ratio of competing parasperm and changes in the amount of seminal fluid protein transferred (24, 25, 27, 30–32). In *Drosophila*, many of these plastic responses appear to be mitigated by an increase in mating duration (21, 29, 33).

The majority of recent work on sperm competition has focused on *Drosophila* (17, 21, 22, 29, 30, 34–36). Many species within this genus have similar typical courtship behaviours, yet varying levels of polyandry (37, 38). This allows for the distinction between response to selection in polyandrous species and monoandrous species (those that mate with only one male), with courtship variables kept relatively constant. Interestingly, some monoandrous species still exhibit plasticity for copulation duration in response to selection, while a seemingly polyandrous *Drosophila* species was recently identified as not increasing mating duration in response to selection (29, 39). As such, the role of plasticity in a male's response to sperm competition appears to be complex.

Previous work on insects and mammals suggests males use several cues to identify their competitive environment. The first of such cues are odorous, organic molecules which are known to affect numerous social and mating behaviours in many animals. Male meadow voles (*Microtus pennsylvanicus*) and the green-veined white butterfly (*Pieris napi*) increase spermatogenic investment (total number of sperm ejaculated) when exposed to pheromone profiles of potential competitor males (40, 41). In crickets (*Teleogryllus oceanicus*), ejaculate investment, determined by the number of

live sperm present in females following copulation, increased when exposed to a competitor male's pheromone-like compounds (cuticular hydrocarbons) at the time of mating (42).

In *D. melanogaster*, males that are rendered incapable of receiving cuticular hydrocarbon signals are still capable of detecting rivals, suggesting that pheromone-like cues are not solely responsible for a competitive response (43). Further work showed the importance of two other sensory pathways: sight and touch. When male *D. melanogaster* are prevented from receiving two signals out of sight, smell and touch from rivals, they are incapable of increasing fertility investment (measured as increased mating duration). Thus, multiple cues can be important in identifying the presence of rivals.

Pheromonal and cuticular hydrocarbon signals are often under high levels of sexual selection and display divergent evolution amongst different species (44–46). As such, the receptors that receive these chemical signals are predicted to also be under divergent evolution. This would suggest that the signals produced by more closely related species should be more similar than those from a more distantly related species, and thus, the male's response to these signals should be stronger when exposed to closely related males than distantly related males. However, responding to competitive males of another species is costly for males as they do not pose a fertility risk; males of other species are incapable of producing viable or fertile offspring according to the biological species concept. Therefore, an alternative prediction is that there should be strong adaptive evolution to eliminate competitive response from those that do not represent a competitive risk (males of different species), regardless of phylogenetic relatedness.

Under this model, only males of the same species should be capable of inducing a competitive response. Unfortunately, this has never been tested experimentally.

I hypothesized that if there is evolution amongst the cues and receptors indicating the presence of competitors, then males exposed to distantly related species should show a weak competitive response, while those exposed to more closely related species should show a medium level response, with the strongest response being to conspecific males. However, if there is adaptive evolution against the response towards males of different species, then there should be no competitive response irrespective of the genetic relatedness of species. To determine which response is most likely, I wanted to assess the response of *D. pseudoobscura* males to conspecific rivals, closely related (*D. persimilis*; diverged approximately 590,000 years ago) and distantly related (*D. melanogaster*; diverged approximately 25-55 million years ago) heterospecific pseudorivals, and compare these competitive responses to males that have developed in the absence of rivals (47, 48). There are several aspects of mating behaviour that I identified: the time it took flies to begin and length of courtship (courtship latency and courtship duration, respectively), as well as the time it took to begin and length of copulation (copulation latency and copulation duration, respectively). However, increases in copulation latency or courtship duration could be a result of increases in female rejection behaviour in response to the male social condition. As such, I wanted to identify if there was a difference in female rejection behaviour of males in response to courtship.

3.2 Methods

3.2.1 Stocks

All flies were maintained at 22±1°C in 8 dram plastic vials containing 5 mL of standard yeast-cornmeal food medium (Bloomington *Drosophila* Stock Center Recipe). *D. pseudoobscura* (San Diego Stock Center line 14011-0121.149) were sampled in San Luis Potosi, Mexico in 2000. *D. melanogaster* flies were generously donated to the lab by Dr. Brent Sinclair after collection in London, Ontario, Canada (2009). *D. persimlis* flies (San Diego Stock Center line #14011-0111.49) were sampled from Mount St. Helena, California, USA in 1998.

3.2.2 Mating Assays

To assess the response of *D. pseudoobscura* males to potential rivals, virgin male flies were aspirated immediately (<2 minutes) upon eclosion and placed either alone in a vial ("isolated condition") or with 9 other males for a total of 10 males in the vial ("competitive condition"). In the conspecific competition environment, *D. pseudoobscura* males were housed with other *D. pseudoobscura* males; in the heterospecific assay, *D. pseudoobscura* males were housed with either *D. melanogaster*, *D. simulans*, or *D. persimilis* males. To reduce vial-related effects, isolated and competitive condition males were collected from the same vials. Males were left to develop in their respective conditions for 5 days. Virgin females to be used for mating assays were also collected immediately upon eclosion to reduce the presence of volatile male compounds they may be exposed to as this has shown to produce male responses. Females were then group housed in the vials for a period of 5 days.

To assess mating behaviour of flies in response to perceived competition, a single male from the isolated condition or competitive condition was placed in a vial containing

5 mL of standard yeast-cornmeal medium that contained a single 5-day-old virgin female. The time until the start of courtship (courtship latency), start of copulation (copulation latency), copulation duration and total time courting (courtship duration) were recorded. A metric for the intensity of mating was tabulated, determined as the total cumulative time spent courting as a fraction of the entire assay.

To assess competitive mating success of males from different sperm competition conditions, one male from either the isolated condition or one male from the competitive condition were placed in a vial with a female, followed by introducing the second male. To avoid confusing the condition to which each male belonged, they were aspirated into the vial one at a time. Since being first in the vial could produce a competitive advantage, the male condition added first was alternated. When one male mated successfully, the competitive condition to which they belong was noted.

To assess female rejection behaviour in response to males based on sperm competition, seven rejection behaviours in response to both isolated and competitive males were scored: flicking (wings flicked in response to male courting), fending (leg extended and maintained), walking away, running away (female quickly moves away faster than male can follow), extending her ovipositor, curling her abdomen inward, and kicking at the male. These were determined by observing typical rejection behaviours exhibited by females. Then males from either condition were placed alone in a vial with a female for 40 minutes or until mating occurred. During the assay, the number of rejection behaviours exhibited by the female was recorded. Males that did not interact with the female in any way for the entire duration of the assay were eliminated from the analysis.

3.2.3 Statistical Analysis

All statistical analyses were conducted in R Statistical Software (2.15.2). Only

individuals who successfully mated were used for analysis of behaviours. Courtship

latency, copulation latency, courtship duration, copulation duration and courtship

intensity (total time spent courting/total time) were assessed for normality using a

Shapiro-Wilk test and for homogeneity of variance using Bartlett's test. Non-normal data

(Copulation Latency, Courtship Duration, Courtship Latency) were square root

transformed and retested for normality. Normal data with equal variance were assessed

using a one-way ANOVA with multiple comparison (Tukey's Post Hoc). Data that could

not be fit to normality were assessed for differences with Kruskal-Wallis analysis of

variance with multiple comparison. Comparisons of mating frequencies and rejection

behaviour frequencies were conducted using Fisher's Exact Test.

3.3 Results

Males reduce their copulation frequency when they are housed with other males

prior to being presented with a female. Compared to when they are housed in isolation,

there is a significant reduction in copulation frequency when *D. pseudoobscura* males are

previously placed with conspecifics (Fisher's Exact Test, $p<0.05$) but not when they are

housed with the distantly related *D. melanogaster* (Fisher's Exact Test, $p=0.1958$) or

when housed with the closely-related *D. persimilis* (Fisher's Exact Test, $p=0.7659$).

D. pseudoobscura males in isolation successfully mated 60.6% of the time (20/33) while

males who developed in competition with conspecifics successfully mated 34.5% of the

time (20/58).   *D. pseudoobscura* males that developed in competition with

D. melanogaster and *D. persimilis* successfully mated 42.3% (11/26) and 68.4% (13/19)

of the time, respectively.

There was also a significant effect of competition treatment on courtship latency

(Kruskal-Wallis test, χ^2_3=33.0093, p<0.0001; Figure 3.1A). As previously shown for *D.*

melanogaster, *D. pseudoobscura* showed a significantly longer time to begin courtship

when developed in competition (Kruskal-Wallis test corrected for multiple comparisons,

p<0.00833, α=0.05). This effect was the same whether they were housed with

conspecifics or heterospecifics (Kruskal-Wallis test corrected for multiple comparisons,

p<0.00833, α=0.05, Figure 3.1A).

There was a significant effect of developmental condition on latency to copulation

(Kruskal-Wallis Test, χ^2_3=40.8715, p<0.0001; Figure 3.1B). This effect is likely an

artefact of significance on courtship latency as there was no significant effect of

developmental condition for the difference in courtship duration, which is the time

between the initiation of courtship and the initiation of copulation (Kruskal-Wallis Test,

χ^2_3=4.8093, p=0.1863; Figure 3.1C). While differences in courtship duration were not

statistically significant, it should be noted that there was a consistent trend of males

having a longer courtship after being housed in a competitive environment compared to

those housed in isolation. Taken together, the above results suggest that there is a

significant increase in the time taken to initiate courtship in the presence of competitors,

and this effect is not dependent on the species present during the time prior to the male's

introduction to a female.

The values for courtship latency and copulation latency (but not copulation
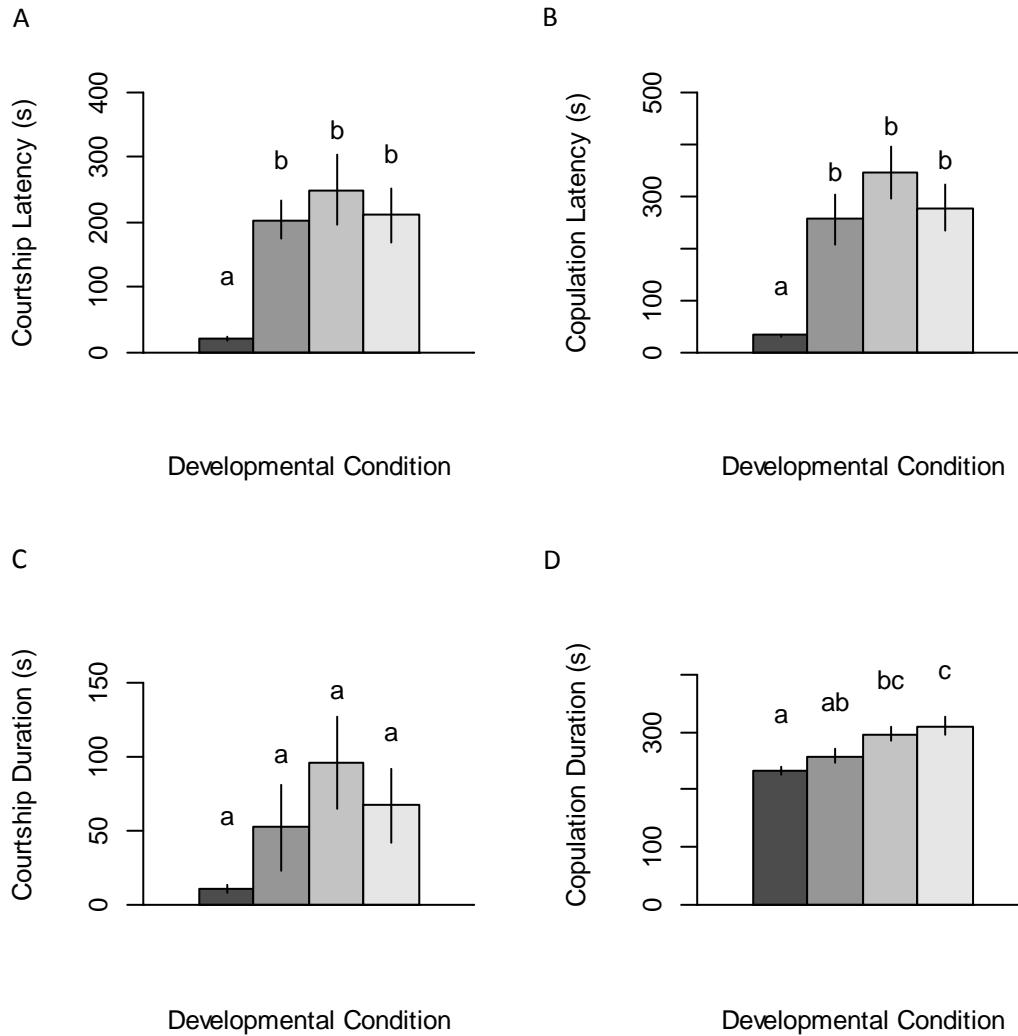
Figure 3.1 *Drosophila pseudoobscura male courtship and copulation behaviour across different social conditions.* Male courtship and copulation behaviour in response to four different conditions: isolated males (n=20), males developed in competition with *Drosophila melanogaster* (n=26), males developed in competition with *D. persimilis* (n=19), and males developed in competition with conspecifics (n=20; left to right in each panel). Four different behaviours were measured as males were exposed to females: courtship latency (A), copulation latency (B), courtship duration (C), and copulation duration (D). Different letters indicate significant difference at $p < 0.05$.

duration) also had a significantly greater amount of variation when males were housed in competitive environments compared to when they were housed in isolation (Levene's Test, $p<0.001$). This is likely simply due to the increased amount of genetic variation present within the competitive assay. For example, different groups of competing males would likely have variation for a variety of traits, including those traits that identify their presence to the focal male. Such variation would be expected to elicit variable responses in the focal male.

As previously shown in *D. melanogaster*, *D. pseudoobscura* males housed in a competitive environment showed a significant increase in copulation duration compared to males housed in isolation (Tukey's Post Hoc corrected for multiple comparisons, $p<0.0001$; Figure 3.1D). The length of copulation duration is also significantly affected by which species of male were added as potential competitors (one-way ANOVA, $F_{3,60}=10.15$, $p<0.0001$). Interestingly, there is a scaled upward trend in mating duration compared to the evolutionary relatedness of the males that are present: males that were housed in isolation copulated for the shortest period of time, followed by those housed competitively with *D. melanogaster*, those housed competitively with *D. persimlis*, and then those housed competitively with conspecifics mating the longest period of time. This trend is further supported by the significantly longer copulation duration *D. pseudoobscura* males exhibit when housed with conspecifics compared to when housed with *D. melanogaster* males (Tukey's Post Hoc corrected for multiple comparisons, $p<0.05$), and when housed with *D. persimilis* males compared to when housed in isolation (Tukey's Post Hoc corrected for multiple comparisons, $p<0.005$).

There was no significant effect of condition on courtship intensity (Kruskal-Wallis Test,

$\chi_3^2 = 0.7736$, p=0.8558).

When isolated males were placed in mating competition assays with males

developed in competition with conspecifics, the isolated male mated first 100% of the

time (n=21). This drastic difference in competitive mating success is likely a male

behaviour and not mediated by female rejection behaviour, as only one of the seven

identified female behaviours (flicking) was significantly more common in competitively

housed males compared with isolated males (Table 3.1).

3.4 Discussion

In this report, I show for the first time that *D. pseudoobscura*, like

D. melanogaster, delay courtship in response to the presence of mating rivals. Previous

reports also indicated the presence of delayed copulation, and I show here that this effect,

at least in *D. pseudoobscura,* is primarily due to the delay in courtship, with only a

minimal contribution of the timing of copulation separate from this effect. Secondly, I

show for the first time that both conspecifics and heterospecifics can induce this delay in

courtship. I further show that, in individuals who successfully mated, the presence of

either closely-related heterospecifics or distantly-related heterospecifics increase

courtship latency to the same degree as the presence of conspecifics.

I also report that the presence of competition increases copulation duration, and

that this increase is inversely correlated with genetic distance amongst the species. This is

the first evidence that plasticity to sperm competition can be induced by heterospecific

Table 3.1 *Number of* **Drosophila pseudoobscura** *females displaying rejection behaviour when exposed to males developed in isolation (iso) or raised in competition (comp).*

	Flicking (frequency)	Fending (frequency)	Walk Away (frequency)	Run Away (frequency)	Extend Ovipositor (frequency)	Inward Abdominal Curl (frequency)	Kicking (frequency)
Iso (n=14)	0 (0)	1 (0.07)	3 (0.21)	6 (0.43)	4 (0.29)	2 (0.14)	2 (0.14)
Comp (n=15)	5 (0.33)	2 (0.13)	6 (0.4)	6 (0.4)	7 (0.47)	2 (0.13)	3 (0.2)
p-value (Fisher's Exact Test)	0.042	1	0.43	1	0.45	1	1

individuals, and further, that this response is potentially scaled by phylogenetic relatedness. This result suggests that males evaluate multiple signals in assessing the presence of competitors, and as species diverge, the signals that indicate the presence of rivals also gradually diverge. It also indicates that the presence of some of these signals is sufficient to elicit a response, and that the response may be scaled based on the quantity or quality of the signal. It is possible that the small number of heterospecifics used in this study produced a scaled response by chance, and that there is no correlation between genetic relatedness and response to sperm competition. Should this be the case, distantly related species should be equally likely as closely related species to contain identical social cues indicating their presence. Evidence from many species indicate that chemical social cues are important for species recognition and are often rapidly evolving between species (44, 45, 49–52). As such, it seems unlikely that there is not a relationship between divergence and recognition of heterospecifics; however, examination of a greater number of heterospecifics would solidify our understanding.

Many reports have surfaced in recent years revealing how males respond to perceived competition when exposed to conspecifics (21–29). Phenotypic plasticity is a common theme throughout many animals as they adjust characteristics that maximize their potential reproductive capacity. For *D. melanogaster*, evidence suggests that plasticity in the presence of competition does enhance number of offspring produced early in life, but these benefits seem to be reduced later in life and also come at the cost of decreased lifespan (17, 22, 34). Due to these costs, the identification of, and response to, rivals should be specific only to those that pose a potential reproductive threat. However, my results show that there is a response by *D. pseudoobscura* males to both

D. melanogaster and *D. persimilis*. In the lab, there has never been a reported case of a mating taking place between *D. melanogaster* and *D. pseudoobscura*. Furthermore, I can predict that such a mating would simply not produce offspring due to strong reproductive isolation and approximately 30 million years of genetic divergence (48). While *D. pseudoobscura* males are capable of producing offspring with *D. persimilis*, the male offspring are sterile and female offspring yield low fertility (53). Since neither of the assessed species pose a strong reproductive threat, it is surprising that *D. pseudoobscura* males are sensitive to their presence and respond in a fashion trending towards that seen for conspecifics.

The response in copulation duration to the presence of heterospecific males yields interesting conclusions on the evolution of rival recognition. Volatile pheromones seem to be an important cue for rival recognition in both mammals and insects (40–42). Males responding to heterospecific rivals may in fact be responding to the presence of particular pheromone compounds or blends. As the surface compounds in *Drosophila*, known as cuticular hydrocarbons (CHCs), evolve, so too do the receptors which receive them (50, 54). Divergence in either signal or reception could lead to a reduced or absent response of *D. pseudoobscura* males towards heterospecific males. One set of compounds in particular that identifies the difference between *D. pseudoobscura* and *D. persimilis* is the ratio of two compounds present in both species: (Z,Z)- 5,9 heptacosadiene and 2-methylhexacosane. Interestingly, 2-methylhexacosane is also present in males of *melanogaster*, but (Z,Z)- 5,9 heptacosadiene is not (55). Thus, it is possible that the presence of either of these compounds on potential rivals could elicit a behavioural response in *D. pseudoobscura*, with the presence of both compounds inducing a stronger

response than the presence of a single compound. Therefore, this difference in pheromone composition amongst the three species could reasonably be perceived as a possible contributor to the difference in response.

Although the likely importance of chemical signals should not be understated, other factors clearly also play a role in identifying the presence of competing males. It is possible that heterospecific males are not inducing differences through CHC profiles, but instead through other mechanisms such as sound or touch, both of which heterospecifics are capable of producing. Both song and CHC profile of *Drosophila* appear to evolve rapidly, and are even capable of species identification, and therefore may be implicated in identification of rivals (44–46, 49, 56).

In the absence of rivals, the male had a qualitatively extreme behavioural response when mating, yet there was only a mild response in the total time spent courting (Figure 3.1C), and there was no significant difference in the intensity of courtship during that time (Figure 3.2). The effect was most identifiable when the isolated males were paired together with competitively-housed males in a competitive mating condition. In these assays, isolated males successfully mated with the female 100% of the time. This seems counterintuitive, as it would imply a higher mating success of isolated males over competitive males. However, females did not increase their rejection behaviour when mating with males that had been housed in a competitive environment (Table 3.1), suggesting the observed plasticity is male-mediated and not female-mediated. There exist several possible non-exclusive explanations for this. First, and perhaps least likely, competitive males are acting in a non-adaptive manner. Since previous studies *Drosophila* have shown a benefit in early life to successfully adjusting mating behaviour,

this seems unlikely (17, 22, 33). Secondly, this could represent an actual strategy employed by males developed in competition to increase their share of paternity in the presence of sperm competition. There is now substantial evidence that, in polyandrous *Drosophila*, males that are the first to mate with a female have a far smaller share of paternity than those that are the second male to mate with the female (57). As such, males expecting competition would benefit from delaying mating until a competing male has already mated. A third possibility is that males developed in competition become choosy about the females with whom they mate. As such, when they are placed with a single female in a no-choice mating assay, they could be reluctant to mate. Lastly, it is possible that this reduction is merely a bi-product of reduced vigor induced by the presence of rival compounds or the interactions with other males. Although the adaptive nature of this last possibility is still debated, there is a large body of evidence that males who are developed in competition show less male-male aggression, as well as reduced male-male courtship(58). It is possible that the neural network responsible for reduced aggression and homosexual behaviour is also involved in male courtship vigour. It would be of interest to explore whether these networks are shared by exploring the male-male aggression behaviour in isolated males and competitively housed males.

In conclusion, the results of this chapter confirm previous results of plasticity in the face of sperm competition in *D. pseudoobscura*. Courtship latency, copulation latency, courtship duration and copulation duration all increased when males were housed with other males. For the first time, I showed that courtship latency, copulation latency, and courtship duration all increased when males were exposed to both recently diverged and distantly diverged heterospecifics. Furthermore, I showed that there was a gradient
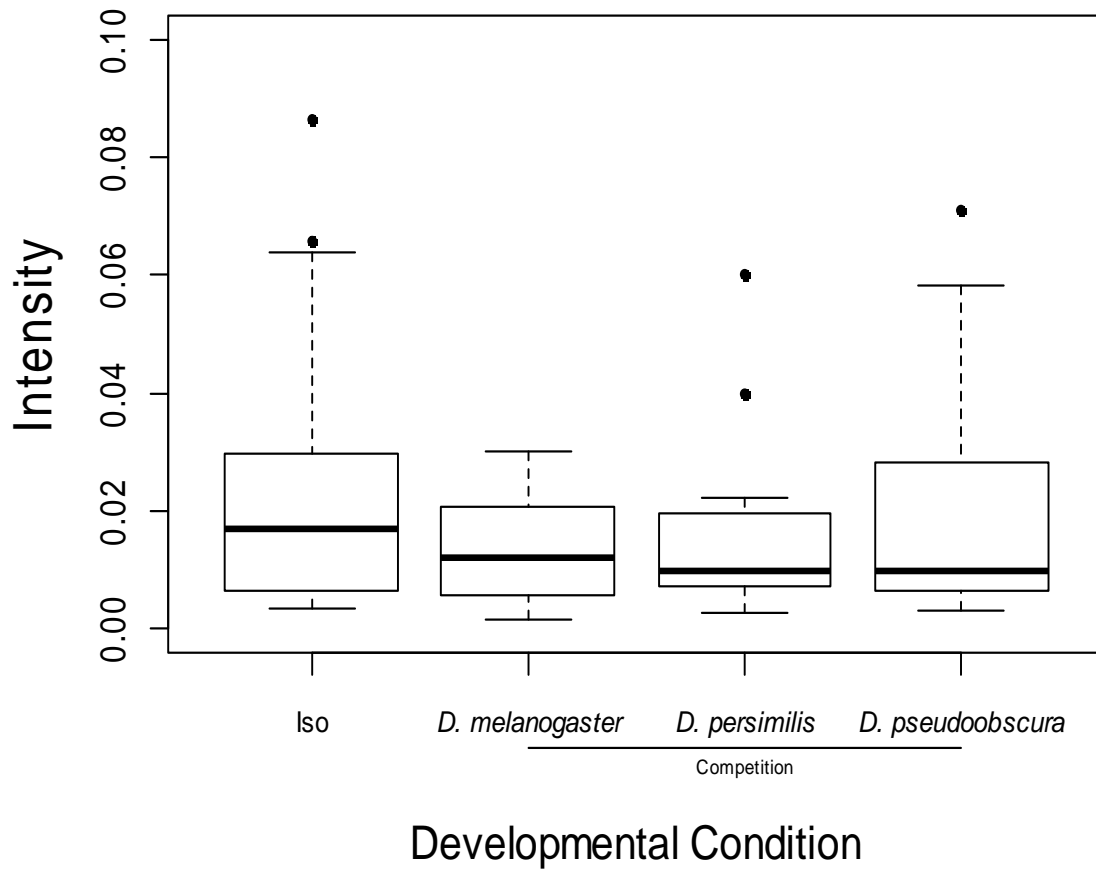
Figure 3.2 *Drosophila pseudoobscura courtship intensity across different social conditions.* Whisker plots where box defines interquartile range. Bars extending beyond the boxes define range with the exception of outliers. Thick middle bar represents median. Dots indicate outliers below 0.1. Two outliers in the Iso condition are not shown at 0.18 and 0.4.

of response for copulation duration from isolated males to when males were exposed to distantly related heterospeciics, closely related heterospecifics, and finally conspecifics. Lastly, I show how these responses are likely not the result of female rejection behaviour, but rather are likely male-mediated effects. Taken together, these results suggest that there is a gradual evolutionary divergence for the production and reception of the signals produced by males conferring the presence of mating rivals.

3.5 References

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Chapter 4

Phenotypic analysis of hybrid sterility & segregation distortion between recently diverged subspecies of *Drosophila pseudoobscura*

4.1 Introduction

Speciation occurs when genetic divergence of populations results in populations that are reproductively isolated from one another (1, 2). Reproductive isolation manifests as either a barrier to the production of hybrid offspring, or when the hybrid offspring are unfit. Hybrid sterility between populations is a common form of reproductive isolation and is one of the first to evolve between populations in geographic isolation (3–8). For nearly all taxa, hybrid sterility of the heterogametic sex (e.g. XY) evolves before the homogametic sex (e.g. XX), a rule known as Haldane's rule.

Through work in *Drosophila* and *Mus musculus*, meiotic drive appears to be an important driver of evolutionary divergence leading to hybrid sterility (9–17). Meiotic drive systems develop initially through a mutation in a gene that distorts allelic inheritance, potentially through killing, inactivating, or preventing the formation of sperm containing competing (non-driving) alleles of that gene (18, 19). In so doing, the meiotic drive element reduces the fitness of the individual by reducing sperm output. Therefore, it is expected that there would be rapid evolution of loci that suppress this meiotic drive element. In lineages that are evolving separately, it is unlikely that the same drive element and suppressor would arise (15, 16, 20). Consequently, if there are divergent

systems in two lineages, hybrids bearing both drive-suppressor systems would display sterility (or reduced fertility) as the meiotic drive systems compete against one another (21).

Much of the work on understanding hybrid sterility comes from genetic analysis of distantly diverged species (2). This confounds speciation studies as it becomes unclear whether the genetic elements producing hybrid sterility today are the same genetic elements that produced hybrid sterility upon its initial appearance. Therefore, hybrid sterility must be assessed in recently diverged populations, subspecies, or species to reduce the confounding divergence since the initial appearance of reproductive isolation.

Though the genetics of hybrid sterility has been the primary focus of speciation geneticists, one surprisingly overlooked area is the specific cytological mechanisms that contribute to hybrid sterility. This is particularly surprising as the cytological basis of spermatogenesis is well established within many species that exhibit hybrid sterility when crossed together (22, 23). In crosses between *Mus musculus domesticus* and *M. spretus*, as well as between *M. m. domesticus* and *M. m. musculus*, spermatogenesis breaks down during meiosis, producing very few, infertile sperm (24–27). In *Drosophila* hybrids, the few studies that have been conducted show that hybrid male sterility manifests during or prior to meiosis (28, 29). Unfortunately, spermatogeneic breakdowns in sterile hybrids of *Drosophila* have generally not been assessed despite a well-defined spermatogenic process.

Two subspecies that are known to produce sterile hybrid offspring are *Drosophila pseudoobscura pseudoobscura* (USA) and *Drosophila pseudoobscura bogtana* (BOG)(30). The USA subspecies inhabits the south western coast of British Colombia

down through to Guatemala (31). It diverged an estimated 150,000-300,000 years ago

from BOG, which exists exclusively in a small radius near Bogota, Colombia (32–34).

These subspecies exhibit F_1 hybrid male sterility only when BOG mothers are mated with

USA fathers. When aged, some of the initially sterile hybrids become weakly fertile, a

phenomenon that has never before been seen for interspecies sterile hybrids (17). The

resulting offspring produced are predominantly female (>90%) when the hybrid is mated

with either pure species or hybrid female.

A single gene (*overdrive*; *ovd*) present on the right arm of the X chromosome is

necessary to cause both hybrid sterility and segregation distortion seen in the hybrids, yet

is insufficient to cause these defects on its own (15, 16). Epistatic interactions with loci

on the left arm of the X chromosome and the second chromosome are required for both

hybrid sterility and segregation distortion, with a minor effect 3rd chromosome locus

necessary for hybrid sterility. The number, location and identity of the genes at these

interacting loci have not yet been identified. Additionally, it is not known where in

spermatogenesis *ovd* acts to disrupt the formation of viable sperm.

In this study, I assessed the spermatogenic basis of hybrid sterility in crosses

between USA and BOG. In both USA and BOG, spermatogenesis begins with a single

stem cell undergoing a self-renewal division, producing a single primary spermatogonium

which is encased in two cyst cells (reviewed extensively in 22). These cyst cells will

encase all spermatogenic products from this one primary spermatogonium until they are

released as mature sperm. The primary spermatogonium will undergo five mitotic

divisions, producing a bundle of 32 primary spermatocytes characterized by drastic

cellular growth (roughly 20X cellular volume)(35). Following this period of growth,

cells enter meiosis, producing 128 haploid spermatids. To accommodate placement of all the mitochondria into the tail of the sperm, the mitochondria aggregate into two connected clumps known as the nebenkern. During this stage, the nucleolus becomes very prominent in the center of each nucleus in the bundle (36). After the nebenkern stage, the sperm tail of all sperm cells begin to form simultaneously as each spermatid elongates along with each nebenkern. The nucleolus fades and the DNA in the cell condenses into a tear-drop shaped mass in each nucleus. Sometime before sperm tail elongation is complete, nuclear elongation begins. Eventually, all 128 sperm are produced in a bundle and released as mature sperm into the testicular lumen. For this chapter of my thesis, I hope to examine which of the aforementioned spermatogenic processes breakdown to produce sterile hybrids.

Based on the results of preliminary studies in other species of *Drosophila*, I predicted that the breakdowns leading to hybrid male sterility would manifest either during meiosis or in premeiotic stages of spermatogenesis. Furthermore, I investigated which hybrid errors were alleviated once fertility is gained in aged hybrids. I hypothesized that if certain spermatogenic errors were alleviated once initially sterile males became fertile, then it is likely that these errors are the ones that are causing hybrid male sterility. The residual spermatogenic errors, if any, still present in these newly-fertile males are not contributing to sterility. Lastly, following the methods conducted previously, I created an introgression hybrid that is homozygous at the *ovd* locus, but heterozygous (heterospecific) throughout the rest of the genome (15). This allowed me to assess *ovd*'s contribution to F_1 hybrid sterility.

4.2 Methods

4.2.1 Fly Husbandry

All species used in this experiment were maintained at 21°C. One *D. p. pseudoobscura* USA line (San Diego Stock number 14011-0121.151) and one *D. p. bogotana* BOG line (San Diego Stock number 14011-0121.168) were kept on ~5mL of standard yeast cornmeal media in 30 mL plastic vials. Newly-eclosed, virgin males and females were separated daily to ensure virginity. Females were kept for a period of 1 week to ensure their virginity (no larvae were produced). F_1 hybrids were produced by crossing virgin USA males with virgin BOG females.

4.2.2 Introgression Crossing Scheme

Introgression of the USA *ovd* region into the BOG genetic background was conducted following the methods of Phadnis and Orr (2009; Figure 4.1). In short, male USA flies containing five visible, recessive X-linked mutations (*ct, se, ll, sp, tt*) were mated with BOG females. Since there is no meiotic recombination in *Drosophila* males, a criss-cross mating scheme was used to reduce the USA content surrounding the *se* locus, which is closely-linked to *ovd*. The *se* locus is 6.5 kb from *ovd*, and only one recombinant was found within 13500 independent lines after 28 generations of backcrossing(15). After 1 generations of criss-cross mating pattern, females containing the *se* locus were mated with males from the original mutant stock. This would produce males that resemble F_1 hybrids with BOG mothers, except ½ will contain the homozygous USA *se* locus, which is linked to *ovd* (F_1^{USAovd}).

Figure 4.1 *Introgression of USA allele of ovd, contained within se, into F1 hybrid.*
Shown are sex chromosomes of flies where long horizontal rectangles are X chromosome
and shorter rectangles with diagonal line attached are Y chromosomes. Solid black
rectangles represent BOG genetic material while white rectangles represent USA
material. Initially, BOG females are mated with USA males containing 5 recessive
visible markers, of which only *se* is of interest. Resulting females inherit a BOG X and a
USA X and are mated with a BOG male. This will yield some males that inherit a
recombined region around *se*. This process is continued for 18 generations, producing
females that have mostly BOG X chromosomes, yet are heterozygous for the region
surrounding *se*. When these females are mated with USA males, two separate hybrid
males are produced: a) males that are genetically identical to USAxBOG F_1 hybrids and
b) males that are genetically identical to USA x BOG F_1 hybrids except they contain USA
alleles around *se*.

4.2.3 Spermatogenesis Analysis

To assess spermatogenic differences between USA, BOG, F_1 hybrid, and

introgression F_1^{USAovd} hybrid males, a combination of phase contrast microscopy and

fluorescent microscopy techniques were used. Premeiotic, meiotic and postmeiotic

cellular structures were observed by extracting five-day-old virgin male testes with

dissecting tweezers in phosphate buffered saline (PBS). Testes were then transferred to a

microscope slide containing 25 mL of 45% acetic acid for 15 seconds. A cover slip was

applied and excess liquid removed. Slides were imaged with a Nikon Eclipse E100

microscope, and analyzed using NIS Elements software.

4.2.4 Hybrid fertility and Segregation Distortion

Timing of hybrid fertility and the offspring's segregation distortion for male F_1

hybrids that have regained fertility and fertile F_1^{USAovd} males was assessed. Males were

placed individually with one female and allowed to mate for ten days. The male and

female were then transferred to a fresh food vial every 7 days. Segregation distortion in

males that regain fertility has been shown to be a product of male hybrids and is not

female-based. As such, this process was continued for the entire life-span of the male.

All offspring from the male were counted and scored for sex until no more offspring

eclosed.

4.3 Results

Premeiotic and meiotic stages of spermatogenesis were identical between pure

species and hybrids (Figure 4.2, 4.3). Differences among the pure species were not

detected until after meiosis, at the nebenkern stage of spermatogenesis. In BOG, nucleoli during the entire nebenkern stage are seen as solid circles under phase contrast microscopy (Figure 4.4). However, in USA, a clearly fragmented nucleolus is present within the nucleus early on in the nebenkern stage (Figure 4.4). As spermatogenesis progresses, this nucleolus appears to reform as one complete nucleolus (Figure 4.4). Both sterile and fertile hybrids contain a combination of both segmented and unsegmented nucleoli early on, yet all remaining segmented nucleoli appear to dissipate later in the nebenkern stage (Figure 4.4).

As the nebenkern elongate, the nucleolus dissipates and genetic material congregates on the periphery of the nucleus. Following this stage, the genetic material condenses near the middle of the nucleus as a tear-drop shaped mass (Figure 4.5A) until nuclear elongation (Figure 4.5B). In wild-type, fertile *D. pseudoobscura*, male mature sperm are achieved by the second day of spermatogenesis. In young sterile F_1 hybrids (<3days old), the nebenkern elongation stage likely represents an arrest in spermatogenesis as later stages of spermatogenesis are not present before males are three days old (Figure 4.5C). By the third day of development, spermatogenesis progresses further where the genetic material congregate in the stereotypically tear-drop shape (Figure 4.5D). On the sixth day of development, sperm are capable of elongating further, and roughly half the nuclei in a bundle elongate (Figure 4.5E).

When sperm tail and nucleus elongation is complete, the sperm are released from the bundle as free sperm. In pure species, these appear as long, string-like structures (Figure 4.6A). However, in F_1 hybrids, only approximately ½ of the sperm achieve this
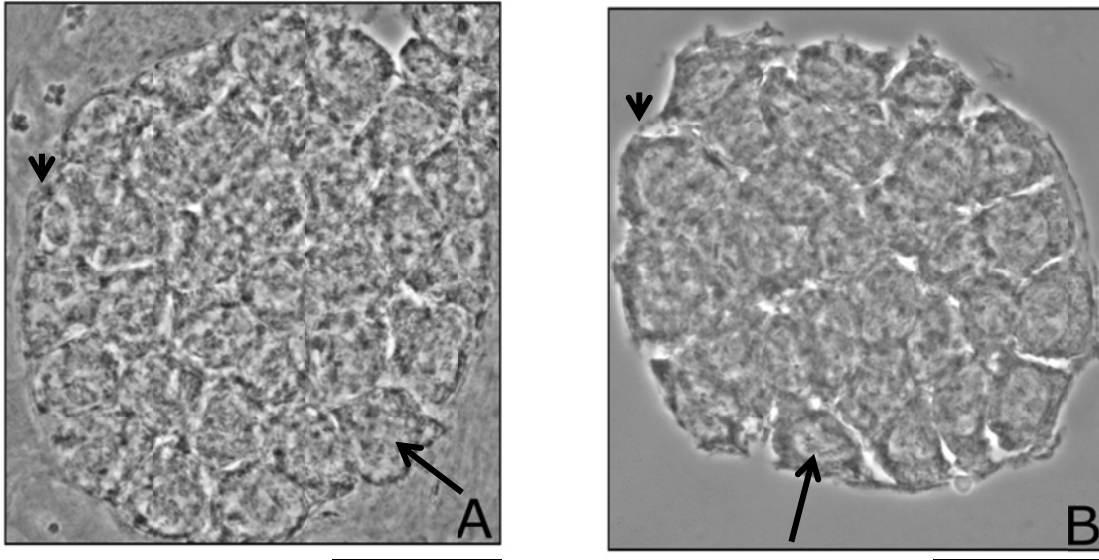
Figure 4.2 *Images of primary spermatocytes (32-cell stage) of spermatogenesis for pure species (A) and F1 Hybrids (B).* At this stage, 32 primary spermatocytes (long arrow) are contained within two cyst cells (short arrow) in a circular shape. Phase contrast microscopy at 1000X magnification. Scale bars are 20 µm.

Figure 4.3 *Images of spermatocytes following meiosis 1 and during meiosis 2.* X- and Y-bearing spermatocytes of pure species (A, C) and F_1 Hybrids (B, D). Sex chromosomes as identified by shape were highlighted within spermatocytes. Spermatocytes in telophase of meiosis 2 for pure species (E) and F_1 Hybrids (F) are shown. All images were taken using phase contrast microscopy at 1000X. Scale bars are 20 μm.

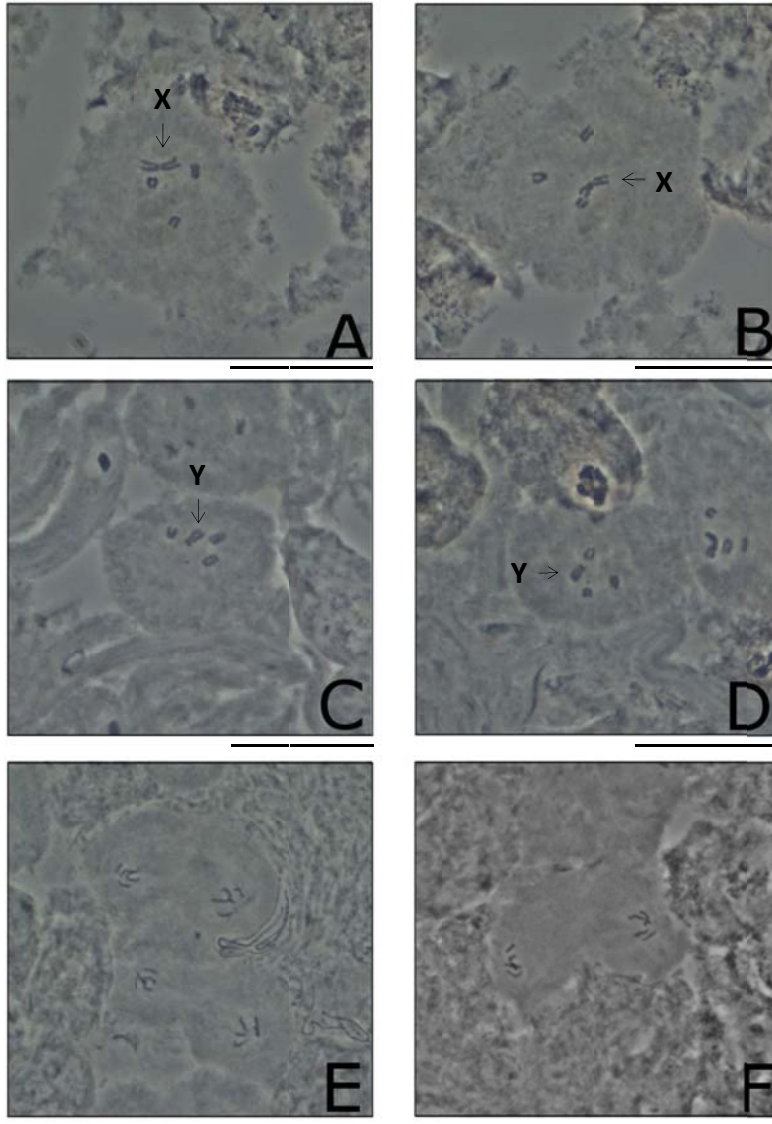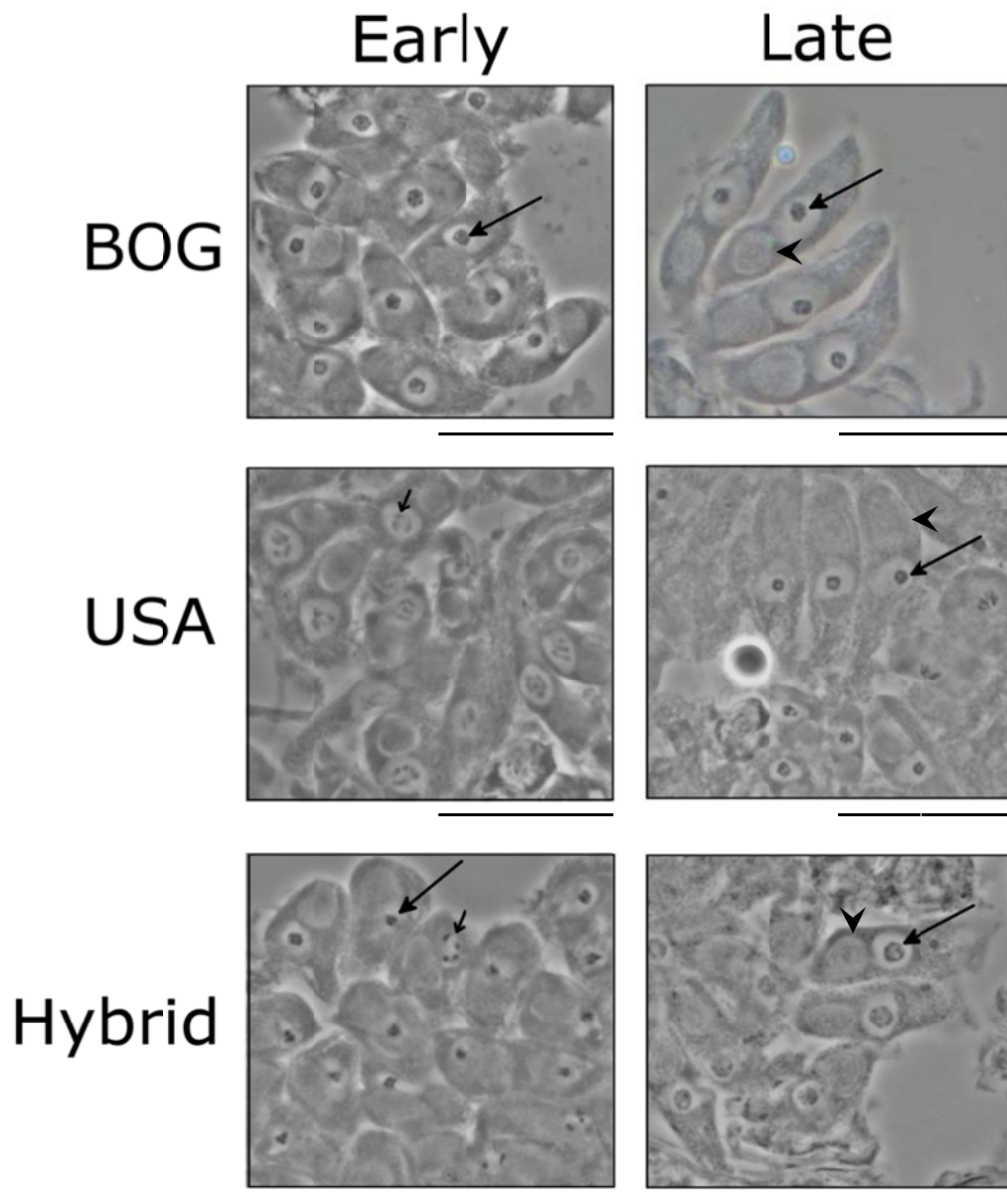**Figure 4.4** *Nebenkern stage for pure species and F1 hybrids compared at both early and late stages.* Both early and late BOG nebenkern-stage spermatocytes show unfragmented nucleoli (long arrow) and a prominent nebenkern (arrowhead). Early USA nebenkern-stage spermatocytes show fragmented nucleoli (short arrow), while late nebenkern-stage spermatocytes show only unfragmented nebenkern (long arrow). Early F_1 Hybrid nebenkern-stage spermatocytes show both unfragmented (long arrow) and fragmented (short arrow) nucleoli, while late staged nebenkern contain only unfragmented nucleoli (long arrow). All images were taken with phase contrast microscopy at 1000X magnification. Scale bars are 20 μm.
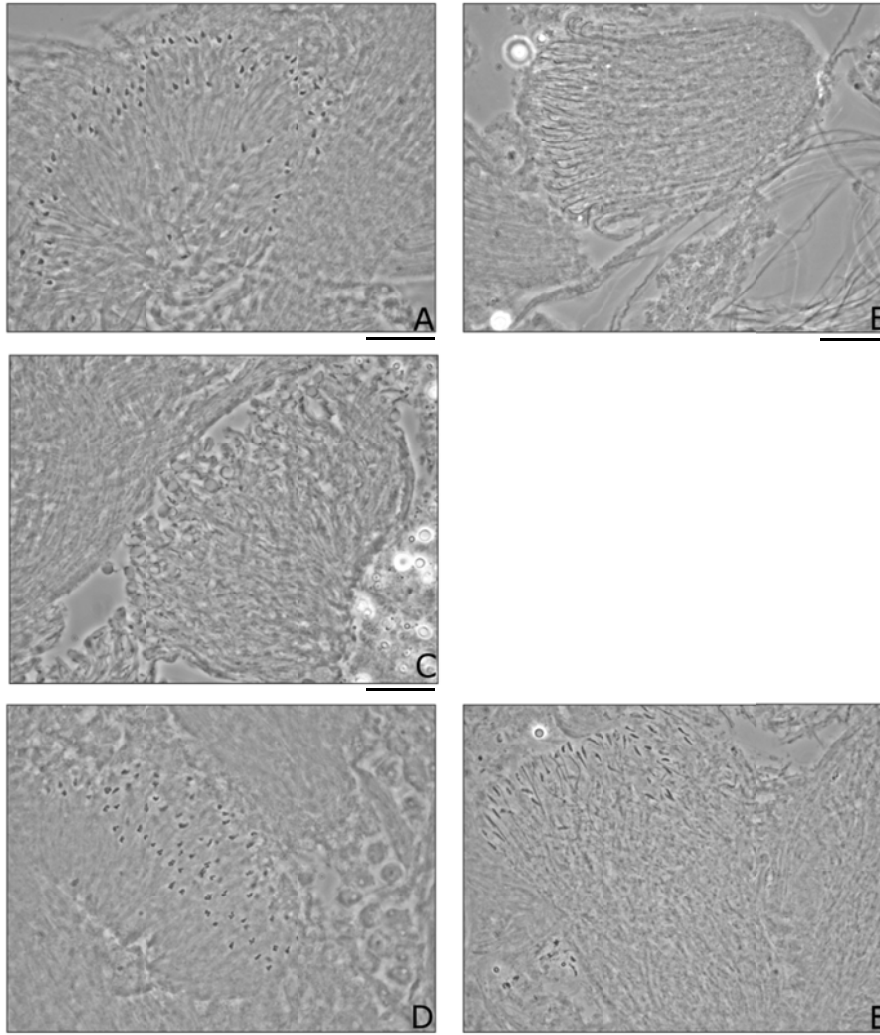
Figure 4.5 *Sperm elongation stage of spermatogenesis for pure species and hybrids.*
Early during elongation, DNA within the nucleus condenses into a tear-dropped shaped
mass in pure species (A). As elongation progresses, the nuclei elongate into thin
structures (B). In sterile hybrids, the spermatid nuclei do not contain condensed DNA
masses (C) and instead appear as empty circles. As the hybrid males age and become
fertile, the typical tear-drop shape is regained in all spermatid nuclei (D). Later in
spermatogenesis (E), these males have roughly half of the sperm in each bundle with thin
nuclei suggesting wild-type elongation, while the other half do not appear to contain
elongated nuclei. All images were taken under phase contrast microscopy at 1000X
magnification. Scale bars are 20 µm.

shape. The other half have several irregular bulges along the length of the tail (possibly incomplete elongation) and irregular nuclear phenotypes (Figure 4.6B, C). Unlike in previous reports of 14 days for fertility to arise, it took just ten days for the hybrids to become fertile (17). The offspring of these fertile hybrids displayed strong segregation distortion with an excess of females in accordance with previous reports (Table 4.1).

All spermatogenic processes for the F_1^{USAovd} introgression hybrids appeared identical to wild-type flies (Figure 4.7). Surprisingly, however, there was still strong segregation distortion in the offspring from F_1^{USAovd} hybrids, which was not significantly different from the distortion produced by aged fertile F_1 hybrids that lacked the *ovd* introgression (Table 4.1). This contradicts with previous findings that showed a normal offspring sex ratio from *ovd* introgressed hybrids (15).

4.4 Discussion

Here, I identified key postmeiotic spermatogenic defects in sterile hybrids of *D. p. pseudoobscura* and *D. p. bogotana*; all premeiotic stages progressed normally. Furthermore, I identified that these defects are alleviated as the sterile hybrids are aged and become fertile. This species subgroup provides the first documented example of postmeiotic failures in spermatogenesis leading to hybrid sterility in *Drosophila*.

Previously, all studies on hybrid sterility phenotypes in *Drosophila* identified premeiotic and meiotic disruptions (28, 29). When F_1 hybrids are made between *Drosophila serido* and *D. koepfera* (estimated divergence 4 million years ago), male
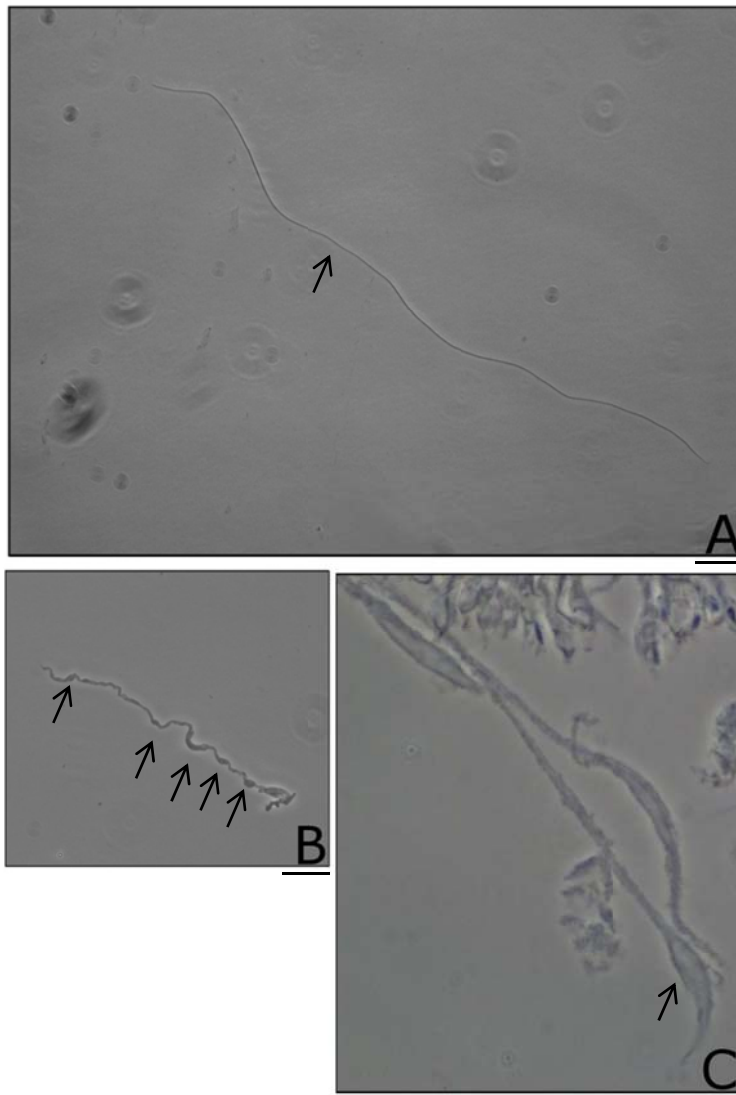
Figure 4.6 *Mature sperm morphs in pure species and hybrids.* Mature sperm of pure species are released from the bundle as thin, string-like structures (A; arrow). In fertile hybrids, ½ of the sperm appear wild-type shape, while the other ½ exhibit irregular elongation patterns and cystic bulges along the length of the tail (B; arrows). The nuclei of these sperm show irregular nuclear elongation (C; arrow). Images (A) and (B) were taken at 400X magnification, while image (C) was taken at 1000X magnification. Scale bars are 20 μm.
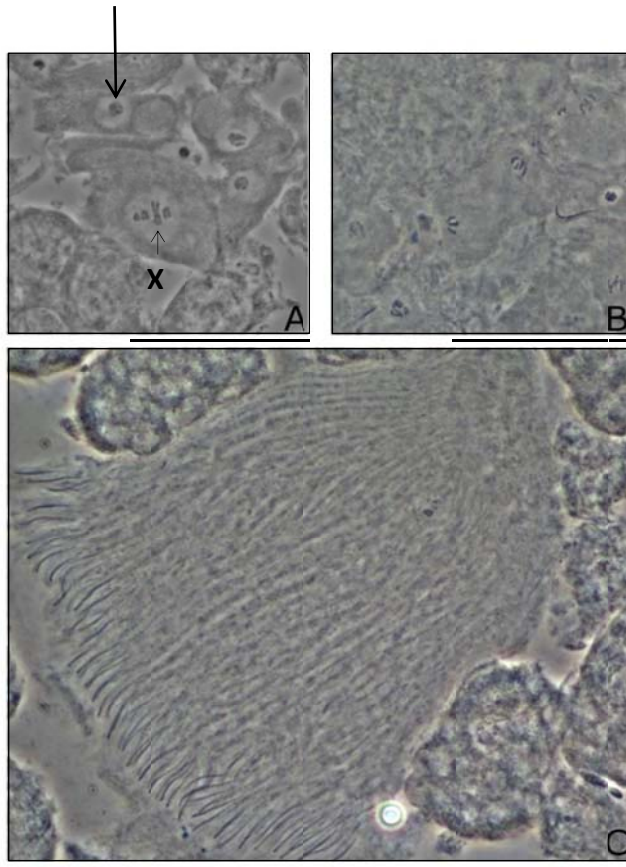
Figure 4.7 *Spermatogenesis of F₁^{USAovd} hybrids.* All stages of spermatogenesis in the introgressed individuals mimic that seen in pure species. Late nebenkern-stage nuclei contain an unfragmented nucleolus (arrow) and wild-type chromosome structure following the first meiotic division as visible through phase contrast microscopy (Short arrow; A). The second meiotic division occurs as in pure species (B). Elongated bundles with nuclei resembling pure species are seen (C). All images are taken at 1000x. Scale bars are 20 μm.

Table 4.1 *Sex ratio of offspring produced from hybrid males.* The first Chi-squared comparison is to the expected sex ratio of 50% females. The second Chi-squared comparison is to the results presented in Phadnis and Orr (2009) for *ovd* introgression males.

Species (n)	Average Sex Ratio (% female)	df	Chi-squared p-value (50% fem)	Chi-squared p-value (94% fem)
USA (10)	52.02	1	0.8875	<0.0001
BOG (10)	54.6	1	0.6711	<0.0001
Hybrids (30)	94.37	1	<0.0001	-
Introgression (75)	90.19	1	<0.0001	0.4343

sterility is caused by the arrest of spermatogenesis prior to spermiogenesis (37). As such, the authors predicted errors took place either during or before meiosis leading to the inability to produce sperm. Crosses between *D. simulans* and *D. mauritiana* (diverged approximately 260,000 years ago) yield sterile males due to failures in meiosis 1 when they have *D. simulans* mothers, and during premeiosis when they have *D. mauritiana* mothers (Alpern *et al.*, unpublished data)(38). In sterile hybrids between *D. pseudoobscura* and *D. persimilis* (diverged approximately 590,000 years ago), F_1 males show non-disjunction during meiosis, predicted through the presence of postmeiotic spermatids with either extra or fewer chromosomes than parental species (Alpern *et al.*, unpublished data)(39). Lastly, sterility in hybrids between *D. arizonae* and *D. mojavensis* (diverged approximately 2 million years ago) is also likely the result of non-disjunction during meiosis, as determined by a bridge connecting currently unidentified chromosomes (Alpern *et al.*, unpublished data)(40).

The aforementioned species all have a greater divergence time than USA and BOG. This would potentially implicate the later stages of spermatogenesis as more sensitive to divergence than earlier stages of spermatogenesis. Therefore, as populations diverge, the initial reduction in fertility of their hybrids would manifest postmeiotically, but as divergence progressed over successive generations, earlier stages of spermatogenesis would also become disrupted in hybrids. The reason for this is not inherently clear. It could be that the later stages of spermatogenesis require the interaction of more genes than the earlier stages of spermatogenesis. As such, divergence at any of the interacting loci could produce sterility at the end of spermatogenesis that would not be seen earlier.

It could also be that the genes contributing to earlier stages of spermatogensis are more constrained than those acting later on; if late-stage spermatogenic genes are more rapidly evolving, then they would be the first to diverge between populations. The observed postmeiotic defect could also be the result of an unobservable premeiotic defect. Many events that take place in spermatogenesis rely on the correct function of premeiotic events. For example, appropriate elongation of the sperm tail and nucleus relies on the appropriate growth of the primary spermatocyte. Should an irregular yet unobservable premeiotic process affect the observed phenotype, such a defect would be seen as postmeiotic. Alternatively, this could merely be a coincidental finding since the number of species that have been assessed for hybrid sterility phenotypes is very low. As such, observing sterility in hybrids between USA and BOG may merely represent a rare case of postmeiotic defects. An extensive analysis of spermatogenesis of sterile hybrids across a larger number of species pairs would help determine which of the above scenarios is most likely.

The introgression of the USA allele at the *ovd* locus fully alleviated observable defects in spermatogenesis. The lack of spermatogenic defects in F_1^{USAovd} hybrids has implications for the function of *ovd* in hybrid sterility: it suggests that *ovd* is the primary causal factor contributing to sterility in F_1 males, and that *ovd* likely induces irregular nuclear elongation in Y-bearing sperm. By removing heterospecificity at the regions linked with *se* (which includes *ovd*) in F_1^{USAovd} males, we showed that all spermatogenic morphology irregularities seen in hybrids were successfully alleviated. These irregularities are thought to be caused by negative genetic interactions between BOG *ovd* with loci from USA (15, 16). A previous QTL analysis identified several X-linked loci

and two autosomal loci that interact with *ovd* to give rise to the sterility phenotype (16). It would be of interest to eliminate heterospecificity at each of these loci in combination with *ovd* to see if different interacting pairs of loci contribute to particular components of spermatogenic failure.

Previous reports have identified overlap between meiotic drive and hybrid sterility loci (10, 12–16). In the offspring of aged, fertile F_1 hybrids, there exists strong segregation distortion causing these males to produce mainly female offspring. This is believed to be the result of a divergent meiotic drive system between USA and BOG. It is thought that a BOG X-linked drive system, which is naturally suppressed by autosomal loci, is unleashed in F_1 hybrids because of a dominant USA allele at the site of suppression. As a result, the drive mechanism is unleashed in fertile hybrids. In this study, I observed that half of the sperm in mature bundles of fertile F_1 hybrids displayed irregular nuclear morphology. This led to the intuitive prediction that these irregular nuclear morphs are Y-bearing sperm that are incapable of proper elongation due to the unleashed drive mechanism, rendering these sperm sterile and leading to the production of predominantly female offspring. Unfortunately, very little information about *ovd* exists limiting the potential to speculate on how it may function to produce the observed phenotypes. Although it is known to function in both hybrid sterility and meiotic drive, the molecular mechanisms have yet to be characterized (15). The protein is predicted to contain a DNA-binding motif and shows a high rate of adaptive evolution in the BOG lineage. Furthermore, expression levels reveal a presence in the testes of pure species and hybrids. This suggests that the transcript itself is functioning aberrantly in hybrids, though the precise mechanism remains unknown.

A previous study showed the absence of sex ratio distortion upon introgression of

USA *ovd* into a hybrid genetic background (15). Surprisingly, I found that the

introgression of USA *ovd* did not alleviate segregation distortion in the study presented

here. The continued presence of segregation distortion is particularly interesting since

my F_1^{USAovd} hybrids have morphologically normal spermatogenesis, including

morphologically normal Y-bearing sperm. There are three possible explanations for the

continued presence of sex ratio distortion in this experiment. The first possibility is that

the difference could be due to the larger amount of introgressed material in my F_1^{USAovd}

hybrids compared to those produced in the original study, which performed 28

backcrosses, reducing the size of the introgression. However, if USA genetic material

induces meiotic drive, then I would have expected to see sex ratio distortion in the

reciprocal cross where the X chromosome is entirely from USA; this was not observed.

Alternatively, there could be recombination between *ovd* and *se*. This seems unlikely as

fertility persisted in the F_1^{USAovd} hybrids, suggesting the two loci have not recombined.

Furthermore, the loci are separated by 6.5kb, making recombination between the loci in

different females unlikely.

The most likely explanation for the continued presence of segregation distortion in

F_1^{USAovd} males is the evolution of more than one meiotic drive system having diverged

between USA and BOG. In the strains used in the original study, one such drive system

was present and could be alleviated by removing the BOG allele of *ovd* (15, 16). In the

strains used in the study presented here, there appears to be an additional drive system,

and this system would still be active even after removal of the BOG allele of *ovd*. There

are now several lines of evidence suggesting meiotic drive and subsequent suppressors

are capable of rapid evolution (19, 21). Furthermore, additional loci beyond *ovd* were

identified in a QTL analysis of segregation distortion (16). As a result, there could be at

least two meiotic drive and suppression systems present within the populations of USA

and BOG, both of which are present in the strains used here. This scenario could be

confirmed through genetic mapping of this additional meiotic drive system.

In conclusion, my results show that spermatogenic abnormalities in the testes are

mitigated as hybrid F_1 males age. Furthermore, I show the sterility defects present in

young F_1 males are eliminated by the introgression of a USA region containing the

speciation gene *ovd*, but that the introgression of this gene does not eliminate the

resulting sex ratio distortion. These results are the first to show an extensive

characterization of the spermatogenic defects that exist in sterile males. They also show

how many meiotic drive mechanisms may have diverged between USA and BOG.

4.5 References

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Chapter 5

Conclusions

For my MSc thesis, I addressed several aspects of reproductive biology in *Drosophila pseudoobscura*. In Chapter Two of this thesis, I looked at the novel classification of parasperm morphotypes in *D. p. pseudoobscura*, and how evolutionary forces of sexual selection in the forms of sperm competition and cryptic female choice have acted to shape the function of these parasperm. In Chapter Three of this thesis, I looked at how the potential for sperm competition has also had an impact on behavioural characteristics important for reproductive success. Lastly, in Chapter Four, I examined the morphological basis of hybrid sterility between two *D. pseudoobscura* subspecies and how it is influenced by the speciation gene *ovd*.

In Chapter Two, I examined how there has previously only been one parasperm morph described in *Drosophila pseudoobscura* (1). I show how this classification is incorrect and how there actually exist two separate parasperm morphs. Parasperm 1 (~50μm) is roughly ½ as long as parasperm 2 (~100μm), which is 1/3rd the length of the eusperm (~300μm). Both parasperm seem to have distinct functions, indicated by an increase in only parasperm 2's proportion transferred upon exposure to pseudorivals. This suggests that parasperm 2 has evolved to function in sperm competition. However, previous reports on the function of *D. pseudoobscura* parasperm have shown that they reduce the detrimental effects of female spermicide, likely by intercepting eusperm

killing compounds (2). By exposing ejaculates to female spermicide, I show that both parasperm 1 and parasperm 2 are equally critical to prevent eusperm death. My results show, for the first time, evidence to support that both sperm competition and cryptic female choice have shaped the evolution of parasperm.

Currently there exists no direct evidence of how parasperm might be functioning in competition. A recent study has developed transgenic fly strains that contain sperm nuclei with green fluorescent protein (GFP) fluorescence (3). This allows for the visualization of sperm movement after they have entered a female`s reproductive tract. Observing the movement and localization of the sperm within the female reproductive tract could elucidate the function of parasperm 2 in sperm competition. The results of this thesis could be supported by parasperm 2 localization to the entrance of sperm storage, blocking the future entrance of rival sperm upon female remating. Furthermore, observing the sperm movement within the female's reproductive tract in real time might reveal sperm behaviour indicative of a role in sperm competition, such as sperm clumping (4). This could allow for identifying novel functions of parasperm 2 in sperm competition.

In Chapter Three, I address how sperm competition has shaped behavioural courtship characteristics to affect how males acquire and mate with females. First, I confirmed previous reports that males respond to the presence of rivals by increasing copulation duration (5–7). I also reported that the initiation of courtship and copulation is significantly delayed in response to rivals. Lastly, I explored the evolution of signals that indicate the presence of rival males by exposing males to different species. I show that closely related heterospecifics induce a significantly longer copulation duration in the

focal male, while more distantly related males induce a weaker response in extending copulation duration. This study shows for the first time how the evolution of male signal production and detection can have an impact on a male's response to the presence of potential sperm competition. This study did not identify the specific cues involved in triggering the competitive response. The critical cues involved in conspecific recognition have previously been studied (8); however, the cues involved in the recognition of heterospecifics has not yet been examined. Cues involved in heterospecific recognition (or lack of conspecific recognition) could be determined through evaluating critical senses previously identified to be involved in the recognition of conspecifics, such as smell, sound, and touch (8). By eliminating the ability of a male to identify individual critical cues given by heterospecific pseudorivals, and subsequently testing the competitive response, the cues involved in heterospecific recognition could be identified. Identification of these cues would tell us how divergent sexual selection can lead to the evolution of species recognition and mate recognition.

In Chapter Four, I focussed on hybrid sterility, a possible outcome of divergent sexual selection, in *D. pseudoobscura*. I analyzed spermatogenesis in pure species as well as sterile and fertile F_1 hybrids between USA and BOG. The results suggested that errors in spermatogenesis of sterile hybrids are exclusively postmeiotic. Sperm tail elongation and nuclear elongation appeared disrupted in all of the sperm produced by sterile hybrid males. When males were aged and regained fertility, sperm tail elongation was no longer disrupted; however, approximately 50% of sperm nuclei within each bundle displayed irregular elongation. Since hybrid males that become fertile produce >90% female offspring, the shortened nuclei were believed to be Y-bearing sperm.

Interestingly, the aforementioned errors in spermatogenesis are not present in fertile *ovd* introgressed hybrids. Instead, there was no observable spermatogenic error despite the continued production of >90% females. Therefore, I conclude that *ovd* was contributing to meiotic drive through the observable spermatogenic disruption phenotype in sterile hybrids. Furthermore, these conclusions suggest there may be several existing meiotic drive systems within *D. pseudoobscura*.

Unfortunately, little functional information about *ovd* exists, limiting the conclusions that can be drawn from this study. However, *ovd* was shown to be expressed in testes of both pure species and hybrids (9). It would be informative to identify the stage of spermatogenesis (see Figure 1.3) in which *ovd* is expressed in pure species by *in situ* hybridization. By comparing the results to hybrids, it would indicate whether there is aberrant expression of *ovd* at a particular stage of spermatogenesis, indicating which stage of spermatogenesis underlies the production of defective sperm. Further information could be obtained through gene knockout and observing the phenotypic outcome. Interestingly, *ovd* is predicted to have a DNA-binding motif at the C-terminus of the putative protein, and is believed to function as a transcription factor (9). Chromatin immunoprecipitation-sequencing (ChIP-seq) could be used to identify the DNA sequence to which *ovd* binds, indicating which genes *ovd* might be regulating. This would also identify any aberrant DNA-binding in hybrids. Lastly, performing a microarray using the male reproductive tract from pure species and F_1^{USAovd} hybrids could identify misexpressed genes in hybrids; pairing this information with the ChIP results could reveal whether genes that *ovd* directly regulates are misexpressed in interspecies hybrids, potentially giving rise to the sterility phenotype.

Sexual selection is predicted to play a strong role in the evolution of reproductive traits (10–13). Parasperm and phenotypic plasticity in the face of competition have been shown to evolve under various competitive conditions (14–20). By re-characterizing parasperm, I was able to show how previous classifications of sperm function were incorrect. Furthermore, through exposure to heterospecifics, I showed that the strength of signals indicating sperm competition are scaled by phylogenetic relatedness. Lastly, I showed how hybrid sterility was induced through postmeiotic breakdowns in spermatogenesis, and how *ovd* contributes to the observable phenotype.

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