Sperm fate and function in reproductive isolation in *Drosophila*

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Central to the study of speciation is the identification of the isolating mechanisms that reduce gene flow. Of special interest are those isolating mechanisms that are the earliest to arise. Sperm fate and function play a major role in two distinct types of reproductive isolation in the genus *Drosophila*. First, postcopulatory-prezygotic (PCPZ) incompatibilities between the male ejaculate and a heterospecific female reproductive tract can result in a reduction in sperm fertilisation success. Although PCPZ incompatibilities have, until recently, received little attention, overwhelming diversity in reproductive morphology and biochemistry indicates that they may play an important role in speciation in this genus. The second type of isolating mechanism that affects sperm occurs in the testes of hybrid males produced by heterospecific matings. These individuals often suffer from dysfunction in spermatogenesis, resulting in the production of aberrant sperm. Hybrid male sterility and hybrid inviability are examples of postzygotic (PZ) reproductive isolation. The observation that hybrid sterility is pervasive among males of all taxonomic groups is known as Haldane's rule. Here we discuss both the evolutionary origins, and functional causes of both PCPZ incompatibilities, which affects sperm fate and function in females, and one type of PZ incompatibility, hybrid male sterility, or sperm dysfunction in hybrid males. Although these two mechanisms of isolation are quite distinct, they are similarly caused by breakdowns in epistatic interactions which occur in the encounter between two divergent genomes. Molecular, cytological, and empirical data are discussed, as is relevant evolutionary theory.

Introduction

Flies of the genus *Drosophila* provide an unusually attractive model system for studies of the reproductive isolating mechanisms that impede gene flow. The genus name *Drosophila* once was used interchangeably with *D. melanogaster,* but the genomes of 12 *Drosophila* species are now sequenced (http://rana.lbl.gov/drosophila) and genetic tools, once only available in *D. melanogaster,* are being developed for other species. Phylogenetic relationships of hundreds of *Drosophila* species are well defined (Markow and O'Grady, 2005a; Remsen and O'Grady, 2002) and the astounding variability among these species in their reproductive biology (Markow and O'Grady, 2005b; Markow, 1996, 2002) reveals that *D. melanogaster* is not representative

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of its many congeners. Furthermore, much of the interspecific and even intraspecific variability in reproductive characters first described by Throckmorton (1962) turns out to have important consequences for the fate and function of sperm once they are transferred to females. Hundreds of *Drosophila* species easily can be reared and used in the laboratory (Markow and O'Grady, 2005a), and many of these species are themselves actually in the early stages of speciation. Thus, *Drosophila* are invaluable resources with which to reveal the mechanisms underlying even the earliest events in reproductive isolation.

A framework widely utilised to explain the genetic basis of the evolution of reproductive isolation is the Dobzhansky-Muller model of incompatibilities (Dobzhansky, 1937; Muller, 1940, 1942) and the snowballing of these incompatibilities with increasing time in the divergence between two taxa (Orr, 1995; Orr and Turelli, 2001). Each of two diverging lineages becomes fixed for contrasting alleles at two different but interacting loci. Each substitution is neutral in its own genetic background, but when they are combined, an epistatic interaction between the two diverged alleles results in an incompatibility of some sort. Given the large number of interacting loci at which mutations can occur and become fixed, the number of potential incompatibilities snowballs with time (Fig. 1). Efforts to understand the very earliest stages of reproductive isolation should therefore focus upon taxa at the earliest stage of divergence.

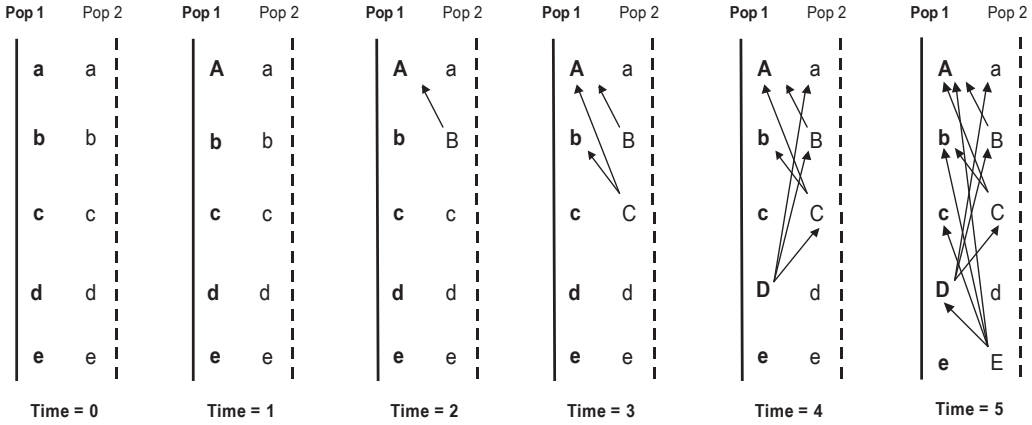

Figure 1. The Dobzhansky-Muller model of incompatibilities. Two populations that initially are genetically identical become separated and over time accumulate fixed changes at different loci. When contact is re-established, the fixed differences may produce genetic incompatibilities that prevent successful reproduction. The number of mutations at different loci increases with time.

Reproductive isolating mechanisms can act at three different levels: precopulatory (PC), postcopulatory but prezygotic (PC-PZ), and postzygotic (PZ). While the Dobzhansky-Muller model is typically applied to cases of postzygotic processes, its central concept, that of incompatibilities between divergent lineages, can be applied to other forms of reproductive isolation (Fig. 2). Sperm fate and function are important components of the last two of these isolating mechanisms: (1) In the absence of complete sexual isolation, interactions between the ejaculate and the female reproductive tract may result in nonrandom sperm storage and or utilization by mated females such that heterospecific or heterotypic sperm are less likely to achieve fertilisation, (2) Should fertilisation and the development of hybrids be successful, a common postzygotic problem is hybrid male sterility (HMS). These two aspects of sperm fate and function will be the focus of this paper.

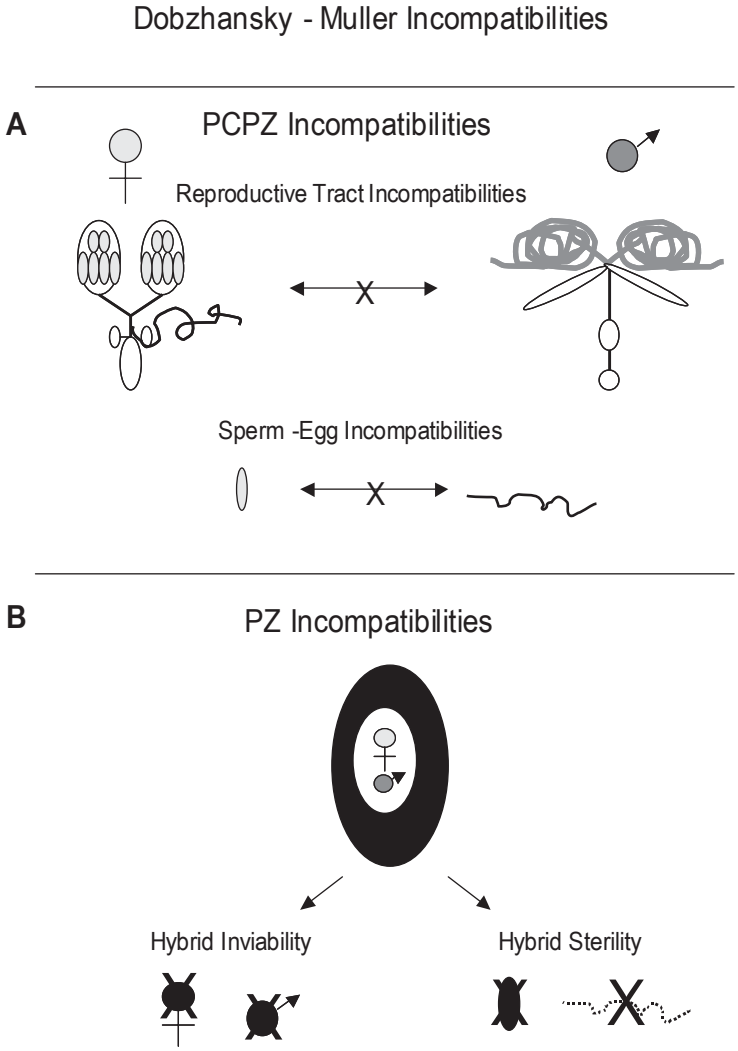

Figure 2. Reproductive isolation through Dobzhansky-Muller incompatibilities at the (**A**) postcopulatory-prezygotic (PCPZ) and (**B**) postzygotic (PZ) stages. PCPZ incompatibilities may occur between the male ejaculate and female reproductive tract or between the sperm and the egg. PZ incompatibilities cause reduced hybrid fitness, through either inviability or hybrid sterility.

PC-PZ processes: The fate and function of sperm in *Drosophila* females

In order to investigate the potential ways in which intersexual reproductive tract interactions can promote reproductive isolation, we first review the reproductive anatomy and biochemistry of both sexes as well as what is known of the processes of copulation, sperm storage and utilization.

Reproductive tract anatomy

Male *Drosophila* internal reproductive tracts consist of a pair of testes each connected to seminal vesicle, paired accessory glands, all connected to an ejaculatory duct and bulb and intromission organ, the aedeagus (Fig. 3). Spermatogenesis occurs in the testes and mature sperm are deposited in the seminal vesicle. Accessory glands produce most of the seminal proteins or accessory proteins (ACPs) passed to females during mating. Testes and accessory glands can vary widely in size and structure in association with ejaculate characteristics. Mature sperm and seminal proteins are combined in the ejaculatory duct, before they are passed through the vas deferens and out the intromission organ to the female.

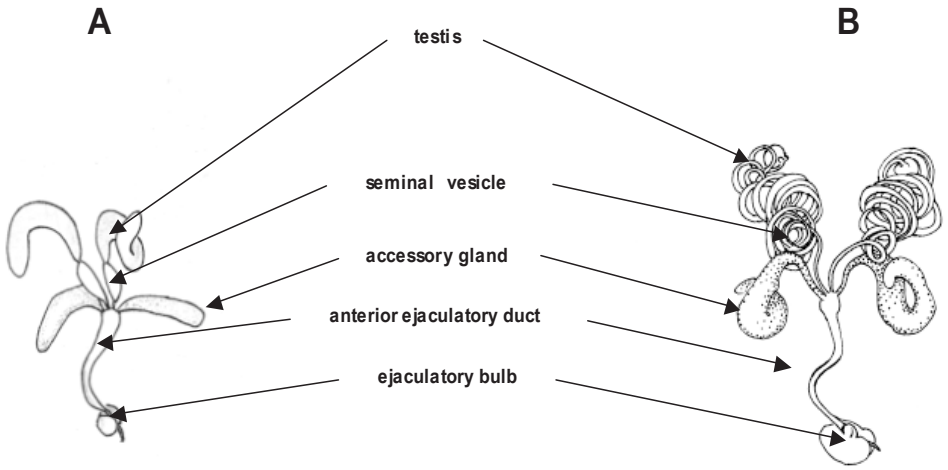

Figure 3. Components of the *Drosophila* male reproductive tract in (**A**) representative of those species producing sperm of a length similar to *D. melanogaster* and (**B**) representative of those species making giant sperm, such as *D. hydei*. Note the length of the testes required to produce giant sperm.

Drosophila female reproductive tracts have paired ovaries connected by lateral oviducts to a common oviduct and eventually the uterus. Ovaries contain ovarioles, long structures in which oocytes are matured from the distal to the proximal portions. Oocytes mature in chambers whose stages were originally classified by King (1970) as 1-14. A stage 14 oocyte is fully mature and ready to be released, fertilised and oviposited. Ovariole number differs markedly among *Drosophila* species, which in turn influences the relative number of oocytes females of the different species produce (Markow, 1996). As in many insects, *Drosophila* females possess sperm storage organs, a ventral seminal receptacle and a pair of spermathecae. In addition to its function in sperm storage, the spermathecal capsule may also generate a secretion important in the maintenance of healthy sperm (Anderson, 1945). The size and morphology of these storage organs are quite variable among species (Fig. 4). Although females of all *Drosophila* species possess both types of storage organs, species differ in whether they use the spermathecae, the seminal receptacle, or both for sperm storage (Pitnick, Markow and Spicer, 1999). Female reproductive tracts also possess parovaria, or accessory glands, whose function has received little attention, but may play a role in fertilisation (Gotteschewski, 1947; Anderson, 1945).

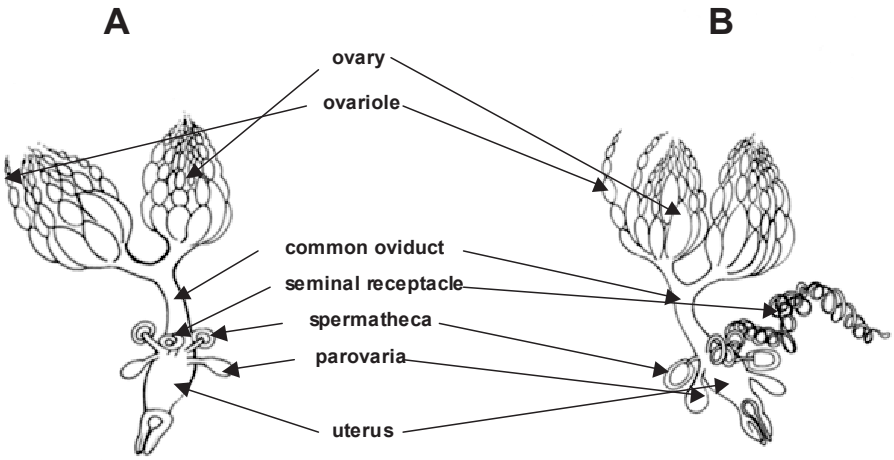

Figure 4. Components of the female reproductive tract in (**A**) representative of species in which males produce sperm of a length similar to *D. melanogaster* and (**B**) representative of species in which males produce giant sperm, as in *D. hydei*.

Reproductive tract biochemistry

Biochemical aspects of reproductive tissue have been more extensively characterised in males than in females, particularly in *D. melanogaster*. Attention has focused largely on the ~80 seminal proteins synthesised in the male accessory glands and subsequently transferred in the seminal fluid to females with the sperm (Wolfner, 1997, 2002, this volume; Kubli, 1992, 2003; Chapman and Davies, 2004). A driving factor in the attention the accessory gland proteins, or ACPs, have received is the discovery that, in *D. melanogaster*, these proteins influence female reproductive behaviour, sperm storage, sperm utilization, and even female longevity (Chapman and Davies, 2004). Accessory glands are assumed to be the primary source of seminal fluid components, but other structures such as the ejaculatory duct and ejaculatory bulb are known to produce at least one protein, glucose dehydrogenase, passed to females in seminal fluid of a number of species (Schiff, Feng, Quine, Krasney and Cavener, 1992; Ross, Fong and Cavener, 1994). Seminal fluid of several insect species has been shown also to contain elements such as sodium (Smedley and Eisner, 1995) and, in *Drosophila,* phosphorus (Markow, Copolla and Watts, 2001) that also are passed to females. For *Drosophila*, little attention has been paid, however, to other seminal fluid constituents, such as carbohydrates, or lipids with the exception of cis vaccenyl acetate (Butterworth, 1969; Brieger and Butterworth, 1970).

Biochemistry of *Drosophila* female reproductive tracts is even less well characterised. Lack of knowledge of the physiology and biochemistry of the tract is probably a reflection of the complexity of this organ system. Genes with enriched expression in the female reproductive tract, which may interact with sperm or accessory proteins, include genes involved in proteolysis, signal transduction, transport, and defense or immunity (Swanson, Wong, Wolfner and Aquadro, 2004). Biochemical characteristics of each organ has not been systematically characterised; however, differences in the expression pattern of one protein, glucose dehydrogenase, suggest that each tissue may have a unique composition (Schiff *et al.,* 1992).

Copulation, sperm storage and sperm utilization

In *D. melanogaster,* in the laboratory, copulations last approximately 20 minutes (Fowler, 1973) and sperm transfer is complete within the first 8 minutes. The additional 10-12 minutes of copulation do not appear to affect male fertilisation success but rather, the time to female remating (Gilchrist and Partridge, 2000). These results indicate that copulation serves a function above the simple transfer of sperm.

After sperm transfer, sperm must enter storage, remain viable, and be released from storage to fertilise eggs. Processes that underlie successful storage and retrieval of functional sperm and their use in fertilisation are highly complex and still not well understood (Nonidez, 1920; Bloch-Qazi, Heifetz and Wolfner, 2003). The fact that certain ACPs often become localised in particular regions of the female reproductive tract suggests that they interact with female tissues and may influence the process of sperm storage and recovery (Ravi-Ram, Ji and Wolfner, 2005). Additionally, changes in gene expression in mated females are indicative of physiological responses that likely play a critical role in mediating post-copulatory processes (Begun and Lawacnizack, 2004; McGraw, Gibson, Clark and Wolfner, 2004).

In *D. melanogaster*, sperm remain in the uterus for several minutes before entering first the seminal receptacle and then the two spermathecae (Nonidez, 1920). This process is known to depend on several components of the male ejaculate, including the lipase *esterase*-6 (Gilbert, 1981; Gilbert, Richmond and Sheehan, 1981; Richmond and Senior, 1981), glucose dehydrogenase (Iido and Cavener, 2004) and ACP36DE (Neubaum and Wolfner, 1999; Bloch-Qazi and Wolfner, 2003). Sperm must be nurtured within the female storage organ until they can be utilised to fertilise eggs. The presence of male-specific lipids in the ejaculate, and elevated lipase activity in both the male and mated female reproductive tracts suggest that fat digestion may be important for the maintenance of healthy spermatozoa (Butterworth, 1969; Smith, Rothwell, Wood, Yeaman and Bownes, 1994). Additionally, both the spermathecal capsule and the parovaria are known to produce secretions that are important for sperm longevity (Anderson, 1945).

Successful fertilisation requires coordination of sperm release from storage with the progression of a mature oocyte through the lateral and common oviducts to the fertilisation site. Seminal proteins, which trigger egg production and ovulation, may assist in this process (Heifetz, Lung, Frongillo and Wolfner, 2000; Chapman, Herndon, Heifetz, Partridge and Wolfner, 2001). Additionally the enzyme glucose dehydrogenase, produced in both the male ejaculatory bulb and the female reproductive tract, has been shown to influence the efficiency of sperm release (Iido and Cavener, 2004). Protein-carbohydrate interactions are important in gamete recognition in deuterosomes (reviewed in Mengerink and Vacquier, 2001), and the presence of glycoconjugates and glycosidases on sperm surfaces indicates this may also be true in *Drosophila* (Perotti and Pasini, 1995; Pasini, Cattaneo, Verni, Hackstein and Perotti, 1999; Cattaneo, Ogiso, Hoshi, Perotti and Pasini, 2002). Two ß-N-hexosaminidases found in sperm cell surfaces are known to be involved in primary sperm-egg interactions (Perotti, Cattaneo, Pasini, Verni and Hackstein,2001). A second type of glycosidase found on the surface of *Drosophila* sperm a fucosidase, is specific to sugar residues found primarily on the micropyle of the egg where fertilisation occurs (Intra, Cenni and Perotti, 2006). How the function of these sperm-egg interactions depends on aspects of the surrounding chemical environment such as pH and ion concentration remains to be examined.

Potential PC-PZ incompatibilities and how they might work

In the preceding section we have described an array of biochemical and morphological interactions that are essential for reproduction in *D. melanogaster*. However, the genus is characterised by incredible diversity of morphological (Fig. 3) and biochemical reproductive traits. Gonad

size, sperm size and sperm number produced by males of different *Drosophila* species show the greatest described variation in the animal kingdom (Pitnick, 1996). Sperm size may be particularly important in male reproductive success, and is under selection in the *obscura* species group (Snook, 1997). Across the genus, sperm size varies by two orders of magnitude, and sperm gigantism has several independent evolutionary origins (Pitnick, 1996; Pitnick *et al.,* 1999). Sperm heteromoprhism, or the production of two sizes of sperm, the smaller of which are fertilisation incompetent (Snook, Markow and Karr, 1994), occurs only in *obscura* group species (Snook, 1997).

Male biochemistry also exhibits extreme diversity. Expression patterns of the gene glucose dehydrogenase, whose role in sperm storage and release is discussed in the previous section, differ between species (Schiff *et al.,* 1992; Ross *et al.,* 1994). The size and presence of an ejaculatory donation, or a protein component of the ejaculate that is incorporated into female somatic and ovarian tissue, is also species specific (Markow and Ankney, 1988; Pitnick, Spicer and Markow, 1997). At the molecular level, male accessory proteins are known to be extremely diverse and some have experienced strong selection (Begun, 2000; Swanson, Clark, Waldrip-Dail, Wolfner and Aquadro, 2001; Kern, Jones and Begun, 2004, Wagstaff and Begun, 2005; Schully and Hellberg, 2006). Additionally, rapid changes in the composition of the male ejaculate have resulted in the presence of many lineage specific accessory proteins (Begun and Lindfors, 2005; Mueller, Ravi-Ram, McGraw, Bloch-Qazi, Siggia, Clark, Aquadro and Wolfner, 2005).

More limited examination of female anatomy and biochemistry indicates they also exhibit divergence of reproductive function between species (Fig. 4). Size, morphology, and patterns of sperm storage organ use are extremely variable (Pitnick *et al.,* 1999). Variation in expression patterns of glucose dehydrogenase within the female tract indicates organ biochemistry and function may also differ between species (Schiff *et al.,* 1992, Ross *et al.,* 1994). The insemination reaction, or an opaque mass that forms in the uterus of certain species after mating, shows both interspecific and interpopulation differences (Paterson, 1946; Wheeler, 1947; Patterson and Stone, 1947; Knowles and Markow, 2001). Additionally, certain secreted proteins and cell surface receptors in the female reproductive tract show signatures of adaptive evolution (Swanson, 2004; Panhuis and Swanson, 2006).

This variation in the reproductive traits of both sexes is indicative of sexual selection that occurs in the reproductive tract of female *Drosophila*; the strength of which may depend on the level of female promiscuity, and therefore the number of competing ejaculates (Markow, 2002). Strong selection predicts divergence between closely related taxa and creates the possibility for morphological and biochemical incompatibilities, as predicted by the Dobzhansky-Muller model, when male ejaculates encounter an unfamiliar female reproductive tract.

Postcopulatory-prezygotic reproductive isolation originating from anatomical or morphological incompatibilities could arise at any of a number of steps following copulation. Optimal reproduction requires a full-length copulation in which both sperm and seminal fluids are transferred. Copulation duration is known to be extremely variable between species ranging from 30 seconds to over 2 hours (Markow, 1996). Interspecific differences in copulation duration, if accompanied by concomitant differences in the nature or number of seminal components transferred could impact the outcome of an interspecific mating. Because male genitalia often are extremely different between closely related *Drosophila* (Markow and O'Grady, 2005b), problems with sperm transfer may be caused by incompatibilities in genital morphology. For example, in crosses between *D. simulans* females and *D. sechellia* males copulations involve the transfer of relatively few sperm. Although most of these sperm later fertilise eggs, the productivity of the cross is significantly reduced when compared to homospecific matings (Price, Kim, Gronlund and Coyne, 2001). Furthermore, if genitalic differences affect the composition or

quantity of transferred ejaculate they could also have implications for the fate of sperm once it is in the female. In heterospecific crosses in the *D. simulans* complex, aberrant copulation durations are associated with abnormal quantities of transferred sperm and/or reduced sperm storage efficiency (Price *et al.,* 2001).

Mismatches between the size or length of sperm and the spermathecae or receptacle could interfere with sperm entering, remaining, or exiting storage organs at the appropriate time. Following artificial selection that modified the relationship between sperm length and ventral receptacle length in different strains of *D. melanogaster,* Miller and Pitnick (2002) found that nonrandom fertilisation occurred. The likelihood of this sort of incompatibility occurring in crosses between natural populations of *Drosophila* is high, given the existence of intraspecific differences in sperm length and ventral receptacle length (Miller, Starmer and Pitnick, 2003). Because fertilisation requires that sperm pass through the egg micropyle, differences in sperm head morphology (Pasini, Redi, Caviglia and Perotti, 1996) may also affect fertilisation success. Though not systematically studied in a large number of species, interspecific variation has been reported for micropyle morphology (Counce, 1959).

The large number of seminal fluid proteins and their roles in sperm fate and the likely importance of their compatibility with the chemistry of the female reproductive tract, suggest that biochemical interactions hold numerous possibilities for reproductive failures. Given the evidence of strong selection on both male and female reproductive proteins, closely related species or even different populations of the same species may not function optimally together. Additionally, differences in the composition of the male ejaculate could mean an essential protein might be absent in an interspecific cross. The failure of just one interaction of the many that must occur between male ejaculate and receptors or enzymes in the female, could mitigate any of these normal postcopulatory processes. Such a failure could be considered as a passive incompatibility resulting, effectively, in a loss of function or signal. For example, Ross *et al*. (1994) demonstrate that while the parovaria of female *D. erecta* and *D. simulans* produce the enzyme glucose dehyrogenase, the other species in the *melanogaster* complex (*D. melanogaster, D. sechellia, D. mauritiana, D. teissieri, D. yakuba)* do not. Given that this protein is known to have an important role in sperm storage and release (Iido and Cavener, 2004), it is easy to envision problems following interspecifc matings with females from species where the parovaria lack this function.

There are several examples of incompatibilities that affect passage, storage, maintenance, and fertilisation success of heterospecific sperm in the genus, although the mechanistic basis of these incompatibilities be they morphological or biochemical remains unknown. A number of interspecific crosses have been examined for the efficacy of sperm storage following single matings. Problems with sperm storage were suggested in an early study of *D. mojavensis* females mated to males of their sister species *D. arizonae* (Baker, 1947). Additionally, reciprocal crosses between *D. mauritiana* and *D. simulans* show a high failure rate in sperm storage (Price *et al.,* 2001). Finally, *D. melanogaster* females fail to store *D. tessieri* and *D. orena* sperm transferred by developmentally modified, mosaic *D. melanogaster* males (Sanchez and Santamaria, 1997). This case is particularly intriguing because the foreign sperm was transferred with *D. melanogaster* seminal proteins, indicative of either a sperm-ACP incompatibility, or a sperm-female incompatibility. Although sperm viability in interspecific crosses has not been explicitly examined in any system, sperm mortality in storage has been reported in many insects including *D. melanogaster* (Snook and Hosken, 2004). Additionally, sperm loss from storage in crosses involving *D. mauritiana* females and *D. simulans* males, severely affects the productivity of this cross (Price *et al.,* 2001).

Stored sperm must also successfully fertilise eggs, which requires temporal coordination of sperm egg release as well as the appropriate biochemical environment for fertilisation. While

no clear examples are known of perturbation of this process in heterospecific crosses, at least two D*rosophila* species exhibit a type of self-sterility or self- incompatibility mechanism in which singly mated females do not utilise sperm from closely related males (Markow, 1997). While the responsible mechanism was not identified, the fact that the sperm of the related male can be "rescued" by seminal fluid of an unrelated male, suggests that the responsible factor is a seminal fluid component (rather than a feature of the sperm) necessary for either oviposition or sperm release and that the responsible factor or factors exhibit genetic variation. This is consistent with the observation that in *D. melanogaster* oogenesis and ovulation are stimulated in mated females by two different seminal proteins acp70A (Chen, Stumm-Zollinger, Aigaki, Balmer, Bienz and Bohlen, 1988; Aigaki, Fleischman, Chen and Kubli, 1997; Nakayama, Kaiser and Aigaki, 1997), and acp26Aa (Herndon and Wolfner, 1995; Heifetz *et al.,* 2000; Heifetz, Vandenberg, Cohn and Wolfner, 2005). An inter- or even an intraspecific difference in the functional properties of such proteins could lead to the inability of a male signal to trigger these processes in mated females. Given that both of these proteins exhibit signatures of adaptive evolution (Aguade, 1999; Begun *et al.,* 2000), these types of incompatibilities seem probable.

Other incompatibilities could conceivably be more "active", blocking sperm movement into or out of storage or the progression of oocytes to the fertilisation site and out the ovipositor. The insemination reaction mass found in certain *Drosophila* species, particularly in the *repleta* group, is known to be more severe in interspecific and interpopulation crosses (Baker, 1947; Patterson, 1947; Knowles and Markow, 2001), affecting oviposition and possibly sperm survival and progression to storage (Baker, 1947; Patterson, 1947). Additionally, the mating plug, a coagulation of seminal proteins which forms in the distal uterus of *Drosophila melanogaster* and *D. hibisci* mated females (Bairati, 1967; Bairati and Perrotti, 1970; Polak, Starmer and Barker, 1998), could also be perturbed in divergent crosses. This possibility, however, has never been examined experimentally.

The biochemical environment of fertilisation and the surface chemistry of the sperm acrosome and the egg micropyle could also have a role in reproductive isolation. Decreased fertilisation success in heterospecific crosses is common in marine invertebrates (Glabe, 1979; Vacquier, Swanson, Metz and Stout, 1999), and depends on molecular interactions between proteins in the sperm acrosome and egg surface (reviewed in Vacquier, 1998). Evidence for similar protein-carbohydrate mediated fertilisation (Perotti and Pasini, 1995; Pasini *et al.,* 1999, Perotti *et al.,* 2001, Cattaneo *et al.,* 2002; Cattaneo, Casini, Intra, Matsumoto, Briani, Hoshi and Perotti, 2006; Intra *et al.,* 2006), and a rapidly evolving seminal protein which interacts directly with sperm (Peng, Chen, Busser, Liu, Honegger and Kubli, 2005) raises the potential for similar fertilisation incompatibilities in *Drosophila*. However, conservation of sperm surface proteins (Intra *et al.,* 2006), and a lack of evidence that sperm-binding seminal proteins interact directly with the egg, provide little support for species-specific gamete recognition in *Drosophila*. The only putative example of a sperm-egg incompatibility occurs when *D. melanogaster* females of the Zimbabwe strain mate with males of a cosmopolitan strain. These heterotypically mated females lay an unusually large proportion of unfertilised or aberrantly fertilised eggs (Alipaz, Wu and Karr, 2001). Whether this is due to problems in gamete recognition, fertilisation environment, or sperm release remains unclear.

When homospecific and heterospecific ejaculates compete in the reproductive tract of a single female, a final class of isolating mechanisms appears. Conspecific sperm precedence (CSP) occurs when the conspecific sperm fertilises a greater proportion of the brood than expected and is well known in many taxa (Howard, 1999). In *Drosophila,* CSP has been reported for the species pairs *D. mauritiana* and *D. simulans (*Price, 1997; Price, Kim, Posluszni and Coyne, 2000), *D. sechellia* and *D simulans* (Price, 1997), *D. yakuba* and *D. santomea* (Chang,

2004) and the subspecies pair *D. pseudoobscura pseudoobscura* and *D. p. bogotana* (Dixon, Coyne and Noor, 2003). However, because sperm viability can have implications for the outcome of sperm competition (Garcia-Gonzalez and Simmons, 2005), CSP may frequently be an artifact of male x female interactions rather than antagonistic interactions between the ejaculates. Only in crosses between *D. simulans* females and *D. mauritiana* males is there evidence that the presence of homospecific semen decreases the fertilisation success of heterospecific sperm (Price *et al.,* 2000).

PC-PZ interactions with the potential to cause isolation actually represent a collection of processes or mechanisms, either anatomical or biochemical, that can occur at one or more of many of the critical steps that lie between copulation and fertilisation. Whether certain classes of interactions, for example anatomical versus biochemical, or storage versus fertilisation, have a greater potential to produce isolation than others, especially early in the speciation process, remains a mystery. Reproductive characters exhibiting relatively greater variability within and between species, such as sperm length, may have a greater potential to cause incompatibilities than less variable traits. As a more complete understanding of all of relevant process underlying the successful transfer, storage, maintenance, retrieval and use of sperm within a species, is generated, our ability to infer how PCPZ interactions can impair normal sperm fate and / or function in interspecific matings will expand.

PZ reproductive isolation: Sperm and hybrid male sterility in *Drosophila*

Haldane (1922) observed that decreases in hybrid fitness are more common in the heterogametic sex. In other words, reduced hybrid fitness will first be seen in the sex with differentiated sex chromosomes (XY, or WZ). This early observation has been verified in a wide range of taxa, including *Drosophila* and mammals where males are XY, but also in birds and Lepidoptera, where females are WZ (Coyne and Orr, 2004, pp. 284-286). Because of the robustness of this pattern, it has become known as Haldane's Rule. Hybrid fitness decrements covered by Haldane's Rule include both sterility and inviability. There is evidence from studies in *Drosophila* that HMS develops before hybrid inviability (Wu, 1992; Coyne and Orr, 1997).

Genetics of spermatogenesis

What causes HMS and what is the nature of the defect or defects observed in sterile hybrid *Drosophila* males? In order to address these questions, we first need to understand normal *Drosophila* spermatogenesis. Fortunately, a good deal is known about spermatogenesis in *D. melanogaster* (Lindsley and Tokuyasu, 1980; Fuller, 1993, 1998). Each testis is a long tube-like structure in which spermatogenesis proceeds from the proximal apex to the distal end, and is joined to the seminal vesicle, where mature sperm wait prior to copulation (Fig. 3). Thus, at the proximal tip of the testis are found the earliest stages of spermatogenesis while mature sperm are found distally. In *D. melanogaster,* it takes 260 hours to go from a mitotically dividing stem cell to a mature, coiled sperm. The process of spermatogenesis is ongoing, continuously, in each testis, or its primordium, from the time of the first larval instar.

For the purposes of genetic studies of male sterility, spermatogenesis can be viewed as occurring in two phases. The first phase includes the premeiotic and the meiotic stages. Spermiogenesis, the second phase, includes nebenkern formation, elongation, individualization, and coiling. A cytological study of 2216 EMS-induced autosomal male sterile mutations in *D. melanogaster* (Wakimoto, Lindsley and Herrera, 2004) revealed that the majority (73%) of the

defects could be localised to spermiogenesis, primarily in elongation, individualization, or coiling. Only 20% exhibited problems in meiosis or earlier, suggesting that later stages of spermatogenesis may be more sensitive to disruption by genetic factors.

Genetics of hybrid male sterility

From studies of male sterility in *D. melanogaster*, we know that a large number of genes, approximately 500, are involved in spermatogenesis and thus have the potential to cause male sterility (Wakimoto *et al.,* 2004). It also appears that some loci affecting male fertility have very few alleles, while others are highly polymorphic. Whether these same genes are those most likely to contribute to HMS is not known. Considering the large number of loci capable of influencing male fertility (Wakimoto *et al.,* 2004), the genetic mechanisms underlying HMS are likely to be complex. Furthermore, evolutionary biologists have tended to utilise rather different approaches than developmental biologists in studying male sterility and its causes.

A critical component in studies of HMS is the way in which it is scored. *Drosophila* developmental biologists have been able to utilise a range of genetic tools, in addition to standard cytological techniques, to fully characterise the defects caused by male sterile mutations. Developmental studies, however, have been restricted primarily to *D. melanogaster* and its relatives. Genetically marked stocks with balancers, important tools of developmental biology, are unavailable in most other *Drosophila* species. *Drosophila* evolutionary biologists have utilised several, more general phenotypic measures of infertility: (1) sperm number (MacDonald and Goldstein, 1999), (2) sperm motility (Coyne, 1984; Vigneault and Zouros, 1986; Coyne and Charlesworth, 1989; Orr, 1989; Orr and Coyne, 1989; Coyne, Rux and David, 1991; Davis and Wu, 1996; MacDonald and Goldstein, 1999; Orr and Irving, 2001; Reed and Markow, 2004; Kopp and Frank, 2005), (3) morphological abnormalities of the testes (Dobzhansky, 1935; Sturtevant and Dobzhansky, 1936; Marin, Pla, Ruiz and Fontedevila, 1993; Joly, Bazin and Singh, 1997; MacDonald and Goldstein, 1999), and (4) the production of offspring (Coyne and Charlesworth, 1989; Marin *et al.,* 1993; Davis and Wu, 1996; True, Weir and Laurie,1996; Tao, Chen, Hartl and Laurie, 2003a, Reed and Markow, 2004). Some studies have examined more than one of these phenotypes simultaneously. While it may seem at first glance to be a discrete or dichotomous trait, sperm motility has been scored in different ways making comparisons among studies difficult. In most cases hybrid males are dissected and sperm scored as motile or non-motile, where the observation of only one motile sperm among many results in a score of motility. Other studies have used a scale or range of motilities, resulting in a more quantitative set of observations (Davis and Wu, 1996; MacDonald and Goldstein, 1999; Reed, LaFlamme and Markow, 2006). Regardless of the scale employed to score motility, the fact that sperm can be motile but functionally sterile (Wakimoto *et al.,* 2004; Reed and Markow, 2004) means that motility cannot be taken as an absolute criterion of male fertility. Reed *et al.* (2006) also found that depending upon whether sperm motility was scored as a binary or graded scale, immotility showed either dominant or additive inheritance, respectively. In addition, studies scoring HMS solely on the basis of offspring production by mated hybrid males may be confounded by reproductive tract interactions of the nature described above.

Observing HMS, regardless of how it is scored, typically has been the endpoint for phenotypic characterisations. The primary question of interest has been assessing the genetic architecture. With few exceptions the actual defects in sterile hybrid males have not been cytologically examined, making it difficult to assess similarities, either phenotypically or genetically, with male sterility found in mutagenic screens of *D. melanogaster*. Kulathinal and Singh (1998) however, in crosses among three species of the simulans clade of the *melanogaster* group,

examined the cytological basis of male sterility. Sterility phenotypes fell into two classes: premeiotic-meiotic and spermiogenic or postmeiotic, with the majority of crosses showing defects of the latter type, much like the proportion of the mutations affecting male fertility in *D. melanogaster* (Wakimoto *et al.,* 2004).

What is known about the genetic architecture of HMS in *Drosophila*? Studies that identify the number of loci contributing to HMS range in their estimates from 3 to 60 (Coyne and Orr, 2004). This range does not appear to correlate with divergence time. For example, three different studies of the species pair *D. simulans* and *D. mauritiana* estimate the number of HMS factors to be 5, 15, and 60 (Coyne, 1984; True *et al.,* 1996; Tao and Hartl, 2003; Tao *et al.,* 2003a; Tao, Zeng, Li, Hartl and Laurie, 2003b). Also, species pairs with different degrees of divergence show the same number of estimated incompatibilities such as *D. virilis*/*D. texana* (D = 0.580, Throckmorton, 1982) and *D. willistoni*/*D. quechua* (D = 0.214, Ayala and Tracey, 1974) which each have an estimated three loci contributing to the male sterility seen in their hybrids (Dobzhansky, 1975; Lamnissou, Loukas and Zouros, 1996). It is generally accepted that HMS is a trait involving multiple genes as has been shown in all studies that have estimated its genetic basis, but it remains unclear how many genes are really involved at the very earliest stages of speciation. One study attempted to estimate the number of loci in two recently diverged subspecies of *D. pseudoobscura* (Orr and Irving, 2001). They found, using a backcross design, that at least 5 genomic regions are involved in HMS at this early stage of divergence. However, because it is impossible to use sterile males in breeding, most investigators resort to introgression and backcrosses in order to dissect the sterility phenotype, breaking up in the process, the very coadapted gene complexes that may be of interest. Genotypes studied above are thus not identical to those of the sterile F_1 males.

An additional complication in most studies of the genetic basis of HMS is the use of mass matings, *i.e.,* placing together many flies from each of two separate populations or species, instead of single pairings from isofemale lines. Use of mass matings precludes detection of early polymorphisms since there is no way to partition any variance in the hybrid male sterile phenotype. Three studies have overcome this problem, however, discovering surprising levels of within-species polymorphism for factors giving interspecific HMS (Reed and Markow, 2004; Kopp and Frank, 2005; Reed, unpublished). From these studies it is clear that in at least three *Drosophila* species, the potential to produce HMS is maintained as a polymorphic trait at varying frequencies in different populations of the same species. In addition the large amount of within population variation observed in consistent with a polygenic basis to the trait.

Another investigation actually sought to identify the nature and number of genetic loci affecting male sterility directly in the F_1 hybrid using within species polymorphism (Reed *et al.,* 2006). They mapped the factors giving HMS within the species *D. mojavensis* in crosses with its sister species, *D. arizonae*. Using quantitative trait locus (QTL) mapping, they found that HMS arises with a complex genetic basis. Since multiple loci were polymorphic simultaneously, it suggests that the loci controlling HMS are likely to accumulate as neutral variants within these species, allowing them to be "caught" in a polymorphic state. Examining species pairs that are early in their divergence trajectories are thus more likely to be successful at capturing the loci where the initial changes arise.

Hybrid male sterility and Haldane's rule

Despite the robustness of Haldane's rule its underlyling genetic mechanisms remain elusive. Several well supported theories attempt to explain Haldane's rule but all lack the necessary ubiquity to explain all of its manifestations. The sex chromosomes play an important role in

many of these theories since it is the heterogametic sex that is disproportionally affected in hybrids. Turelli and Begun (1997) considered whether the size of the X-chromosome influenced the rate of evolution of Haldane's rule across a variety of *Drosophila* species pairs. They found that the strength of Haldane's rule at early stages of divergence was positively correlated with the size of the X-chromosome but the effect was only relevant to HMS. Hybrid inviability and female sterility showed no correlation with X-chromosome size. In addition, the "large X-effect" has been shown in a variety of introgression studies and hybrid zone studies, where the X-chromosome shows greater influence on apparent hybrid incompatibilities than do the autosomes (Coyne and Orr, 2004, p. 290).

There are two prominent explanations for the large role of X-chromosome in Haldane's rule. First, dominance theory (Muller, 1940, 1942) predicts that the X-chromosome carries recessive alleles that only express their Dobzhansky-Muller incompatibilities when in a hemizygous state. This theory has been shown to be consistent with the pattern of hybrid inviability in *Drosophila* when hybrid females are made homozygous for the X-chromosome, thus allowing the expression of the recessive alleles (Orr, 1993; Coyne and Orr, 2004, p. 289). But, this theory does not explain hybrid sterility because hybrid females with the homozygous X do not necessarily show a loss of fertility. This implies that genes causing hybrid inviability affect both sexes while genes involved in sterility are sex specific. Surprisingly, this finding is not predicted by Wakimoto *et al.* (2004) where nearly 40% of the EMS-induced sterile male mutants also showed female sterility.

Another theory for Haldane's rule is called the "faster-X theory" which hypothesises that genes on the X-chromosome are evolving at a faster rate than those in the rest of the genome. The evidence for this hypothesis remains inconclusive. First, when the number of HMS factors on the X-chromosome in the *simulans* clade is estimated, different studies find dramatically different results. Hollocher and Wu (1996) find no difference between the X and autosomes for the proportion of HMS factors, while others find there are more on the X (50%, True *et al.,* 1996; 250%, Tao *et al.,* 2003a). In addition, there is weak evidence for an accelerated rate of evolution of X-linked genes in general (Betancourt, Presgraves and Swanson, 2002; Counterman, Ortiz-Barrientos and Noor, 2004). Finally, despite the evidence for a large X-effect and more HMS factors on the X-chromosome, there is a paucity of sex-linked male somatic and testes specific genes (Parisi, Nuttall, Naiman, Bouffard, Malley, Andrews, Eastman and Oliver, 2003). These results present a quandary, why is the X-chromosome so important if it presents so few targets for mutations causing HMS? Little work has expanded this analysis to other taxa. In one *Drosophila* species pair, however, *D. arizonae*/*D. mojavensis*, no evidence for a disproportionate effect of the X-chromosome has been found (Zouros, 1981; Zouros, Lofdahl and Martin, 1988; Zouros, 1991; Reed *et al.,* 2006). Caution should therefore be exercised when making generalizations about the genetic basis of Haldane's rule and about HMS specifically until the patterns reviewed here are substantiated in other systems.

The "faster male" theory is another, non-mutually exclusive, explanation for Haldane's rule but it is only relevant to heterogametic hybrid males. The idea here is that genes underlying HMS may simply evolve faster, developing incompatibilities more quickly than those for hybrid female sterility. True *et al.* (1996) found that in crosses with *D. mauritiana* and *D. simulans*, far more genetic regions caused HMS than hybrid female sterility. Additional studies have confirmed this pattern in the other members of the *melanogaster* species group (Hollocher and Wu, 1996; Tao *et al.,* 2003; Tao and Hartl, 2003).

In *Drosophila melanogaster* group species male-specific genes have an elevated substitution rate (Zhang, Hambuch and Parsch, 2004) and male specific genes diverge more quickly in expression patterns between *D. melanogaster* and *D. simulans* (Ranz, Castillo-Davis, Meiklejohn

and Hartl, 2003). Further evidence for the faster male theory is the patterns observed in aberrant gene expression in hybrids. In hybrids between two *Drosophila* species pairs (*D. simulans*/*D. mauritiana* and *D. persimilis*/*D. pseudoobscura*), male specific genes show greater perturbation relative to their parental species than either female specific or non-specific genes (Michalak and Noor, 2003; Noor, Michalak and Donze, 2003; Michalak and Noor, 2004; Noor, 2005; Ranz, Namgyal, Gibson and Hartl, 2004). Some of those genes showing aberrant expression patterns in hybrids also reflect increased rates of protein evolution (Noor, 2005). One piece of conflicting evidence for this pattern is that when three genes specific to sperm development were tested for rates of evolution in *D. melanogaster* and *D. simulans*, only one showed evidence for adaptive evolution (Civetta, Rajakumar, Brouwers and Bacik, 2006). Thus, based on this limited sample it is not clear that sperm development is a specific target for the evolution of HMS factors.

Issues of experimental design, especially scoring of male sterility, use of introgression, mass versus single pair mating, and degree of divergence between populations or species being crossed all have substantial impacts on the outcome and interpretation of HMS studies. Furthermore, a preponderance of *Drosophila* investigations have been confined to a small number of species and their relatives, largely those of the *melanogaster* group no doubt owing to the availability of genetic markers. While these studies point to a major effect of the X chromosome, studies of other *Drosophila* species are not characterised by significant X-chromosome effects leaving this generalization in need of additional experiments across a wider range of *Drosophila* species.

Conclusions

Sperm are critical to reproductive isolation at two levels: postcopulatory but prezygotic and postzygotic. We have shown that both of these types of reproductive isolation are complex and depend upon multiple pathways and processes. Incompatibilities may stem from changes either in protein coding genes, as exemplified by substitutions in accessory gland proteins, or by divergence in regulatory mechanisms, as reflected in the differences in tissue specific expression of the enzyme glucose dehydrogenase. While the Dobzhansky-Muller model has been strictly applied to postzygotic isolation, because of epistatic interactions, its central concept, one of incompatibilities accumulating through time, can be applied to different categories of prezygotic interactions between the sexes. Hybrid male sterility is likely to be more tractable for genetic studies as the gross phenotype is largely rooted in spermatogenesis, a process that has been intensively characterised genetically and phenotypically in *D. melanogaster,* and one in which the pathways are likely to be conserved. Postcopulatory-prezygotic impairments of sperm fate and function can occur at a range of steps, none of which are well understood even in homotypic matings. Given the complexity of postcopulatory-prezygotic processes, however, this category of reproductive isolation may turn out to be of major importance in speciation.

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