CHARACTERIZATION OF HYBRID MALE STERILITY IN *DROSOPHILA*

CYTOLOGICAL CHARACTERIZATION OF HYBRID MALE STERILITY AMONG SIBLING SPECIES OF THE *DROSOPHILA MELANOGASTER* COMPLEX

By

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Abstract

Hybridizations between species of the *Drosophila simulans* clade produce fertile females but sterile males. In this study, a comprehensive cytological characterization was undertaken on the six F_1 males that were the result of the crosses between *D. simulans*, *D. sechellia*, and *D. mauritiana*. With the use of light and electron microscopy, it was shown that each particular hybrid genotype exhibited a consistent and characteristic sterility phenotype. The hybrid sterility phenotypes fell into two distinct classes. The two hybrid genotypes that possessed *D. mauritiana* X-chromosomes contained spermatogenic defects that were solely premeiotic in nature. Testes originating from these hybrids enclosed abnormally large cysts of one to eight irregular shaped spermatocytes. The other four F_1 hybrids possessed *post*meiotic spermatogenic defects in the elongation and individualization stages of spermiogenesis. Nonsynchronous cell divisions, underdeveloped mitochondrial derivative-axonemal associations, and microtubule abnormalities were common to all of these hybrids. However, each particular hybrid of the postmeiotic class genotype also demonstrated characteristic consistences such as similar sperm bundle numbers in addition to consistent spermiogenic arrests in spermatids that have developed normally the furthest. The predominance of postmeiotic abnormalities over premeiotic ones indicate the close relatedness

between species of this clade. The large difference between premeiotically defective hybrids bearing *D. mauritiana* X-chromosomes and those hybrids containing postmeiotic defects may imply the existence of a higher rate of evolution on spermatogenic loci on the X-chromosome of *D. mauritiana.* In addition, significant differences in sterility phenotypes between reciprocal crosses (asymmetry) were observed. Hence, the transition from non-reciprocal, one-way, hybrid male sterility to asymmetrical reciprocal hybrid male sterility seems to be normal. The discovery of a locus (loci) originating from *D. simulans-2119,* which caused a large shift in sterility from postmeiotic to premeiotic when crossed to *D. sechellia,* may validate the presence of genes with large effect in the evolutionary progression of hybrid male sterility. Utilizing F_1 hybrids from an attached-X line, it was also shown that the influence of the cytoplasm, originating from *D. simulans*, on hybrid male sterility was insignificant in hybrids of the *simulans* clade.

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Introduction

> "When hybrids are produced by the unnatural crossing of two species, the reproductive system, independently of the general state of health, is affected by sterility...the external conditions have remained the same, but the organisation has been disturbed by two different structures and constitutions having been blended into one."
>
> Charles Darwin (1859), *On the Origin of Species*

The origin of species, historically deemed as the "mystery of mysteries", has persisted to be an enigmatic challenge. An accumulating volume of studies, however, are revealing a number of apparent patterns in the process of cladogenesis. One well documented motif has been the occurrence of interspecific hybrid sterility as a common postzygotic reproductive isolation mechanism among closely related species in diverse taxa of both plants and animals. The biological species concept (Mayr, 1959) has defined the role of hybrid sterility as a fundamental indicator in the systematic separation of closely related species.

The evolutionary importance of hybrid *male* sterility has been exhibited in many genera such as dipterans and mammals. In fact, almost three quarters of a century ago, J.B.S. Haldane produced the empirical statement, "when in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic] sex" (Haldane, 1922). This rule seems to adhere more strongly to sterility than viability (Wu *et al.*, 1992). Departures from the fertility aspect of this rule have indeed been rare

(Table 1.1) especially among *Drosophila*, where males are the heterogametic sex (Bock, 1984). Thus the primary exposure of hybrid male sterility, early in the evolution of two genetically diverging species (where the heterogametic sex is male), has marked it as the first step in the process of speciation (Coyne & Orr, 1989). Ever since Haldane's classical assertion, many attempts at elucidating its genetic basis have been undertaken (Haldane, 1932; Dobzhansky, 1936; Coyne, 1984; Coyne & Kreitman, 1986; Coyne & Charlesworth, 1986; Vigneault & Zouros, 1986; Orr, 1987; Khadem & Krimbas, 1991; Zeng & Singh, 1993). However, the notion that Haldane's rule may not have one single genetic explanation, but is rather a 'composite' rule depending on which particular genus it is applied to, has recently been suggested (Wu *et al.*, 1992; Turelli & Orr, 1995).

While a great deal of effort has been spent in determining the genetic basis of this species isolating mechanism, the actual 'phenotype' of hybrid male sterility has typically been ignored. From Dobzhansky's method of measuring testis size (1937a), to the prevailing method of detecting the presence of motile sperm (Zouros, 1981; Coyne, 1985), existing fertility assays do not allow for the demonstration of specific 'causes' o of sterility. These studies constrain the trait of fertility to be classified as an all-or-none phenomenon. However, different sterile hybrids may possess their own characteristically unique spermatogenic aberrations. And such differential degrees of sterility between reciprocal interspecific crosses, as well as the observed pattern of sterility asses amongst different F_1 hybrids, may invite novel insight into the evolutionary succession of hybrid male sterility.

Table 1.1 Empirical evidence for Haldane's Rule[a]

Taxon	Heterogametic Sex	Trait (Fertility[b]/ Inviability)	Total Asymmetric Hybridizations[c]	Percentage Obeying Haldane'sRule
Drosophila	male	fertility	202	98.5 %
		inviability	23	60.9 %
Mammalia	male	fertility	25	100 %
		inviability	1	0 %
Aves	female	fertility	30	100 %
		inviability	23	70 %
Lepidoptera	female	fertility	15	100 %
		inviability	40	90 %

[a] The data in this table was compiled by Wu & Davis (1993)
[b] Cases whereby both sexes were viable
[c] Cases whereby only one sex is sterile or inviable

In this study, eight sterile F_1 hybrid males (six 'natural' hybrids plus two hybrids from attached-X line crosses) from three sibling species of the *Drosophila melanogaster* subgroup have been characterized for spermatogenic defects. The role of the cytoplasm on hybrid male sterility will also be examined.

1.1 The species

The *melanogaster* subgroup includes four sibling species, *Drosophila melanogaster*, *D. simulans*, *D. sechellia* and *D. mauritiana*, that are morphologically identical except for conspicuous differences in the posterior process of their genital arches (Sturtevant, 1919; Tsacas & David, 1974; Tsacas & Bächli, 1981). Members of the *melanogaster* complex are identical in karyotype as well as in chromosomal banding pattern, with the exception of a major autosomal inversion between *D. melanogaster* and its three sibling species (Horton, 1939; Lemeunier & Ashburner, 1976).

Both *D. melanogaster* and *D. simulans* are cosmopolitan human commensals that are located worldwide. Their origins have an African root, although *D. simulans* seems to have globalized the earth more recently (Nei *et al.* 1975; Singh *et al.* 1986). On the other hand, *D. sechellia* and *D. mauritiana* are island endemics situated respectively on the Seychelles Islands and Mauritius. The Seychelles Islands are a small archipelago of islands situated over 1 000 km northeast of Madagascar while the volcanic island of Mauritius is found a comparable distance due east off the Madagascar coast. *D. melanogaster* and *D. simulans* are not found sympatrically with these insular species (Tsacas & David, 1974). *D. sechellia* is a specialist which resides on its host plant, *Morinda citrifolia*, whereas the generalist

features of *D. mauritiana* allow it to survive on a number of different hosts (David *et al.*, 1987).

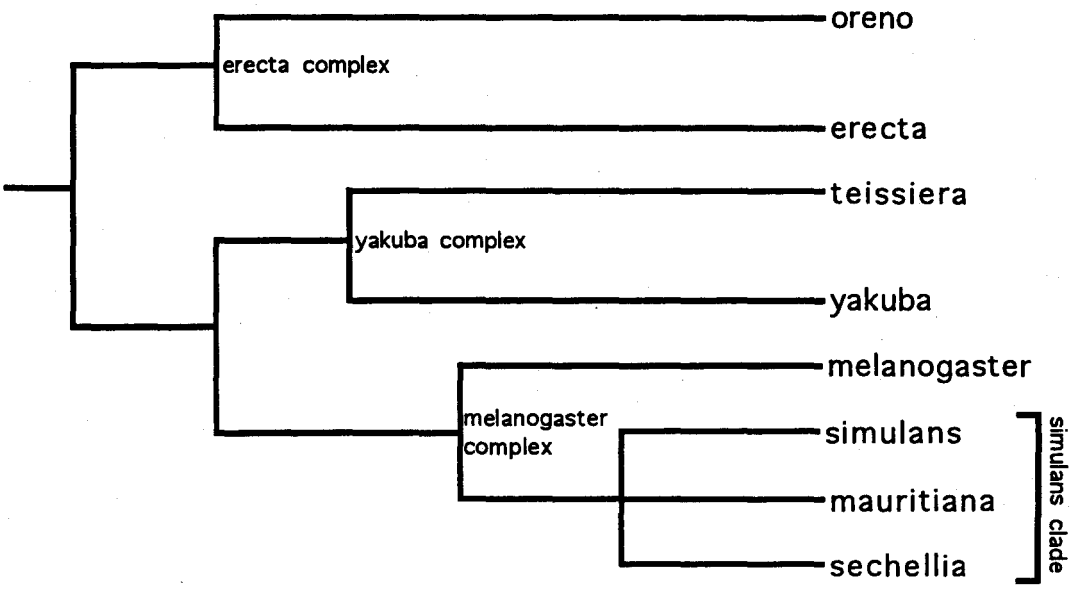
Numerous studies have investigated the phylogenetic relationship between these four species. The apparent disagreement between various character trees has appointed the *melanogaster* complex with an unresolved trichotomy consisting of the '*simulans*-like' species, *D. simulans*, *D. sechellia*, and *D. mauritiana* (Lachaise *et al.*, 1988). Amongst the *simulans* clade, *D. melanogaster* has consistently been placed as the outgroup (Figure 1.1). The three possible *simulans* clade topologies can be explained by an assortment of different characters. The coupling of *D. sechellia* and *D. mauritiana* from a *D. simulans* ancestor (Figure 1.1a) has been based on such evidence as male genitalia morphology (Coyne & Kreitman, 1986), *Adh* and *per* DNA sequences (Coyne & Kreitman, 1986; Kliman & Hey, 1993) and mitochondrial DNA sequences (Solignac & Monnerot, 1986).

Although the early divergence of *D. simulans* from its two sibling species seems to be the most parsimonious phylogenetic explanation, in concordance with the diverse spectrum of evidence, other historical reconstructions remain possibilities. An early *D. sechellia* divergence topology (Figure 1.1b) can be substantiated by evidence comparing reproductive isolation (Lachaise, 1986) as well as phenetically through measures of sperm cyst length (Joly, 1987). And a third topology, representing a recent *D. sechellia* / *D. simulans* separation from a *D. mauritiana* ancestor (Figure 1.1c), rests solely on allozymic data testimony (Cariou, 1987).

Figure 1.1 ***Drosophila melanogaster*** **phylogeny**

The phylogenetic relationship between members of the *Drosophila melanogaster* subgroup. Many different characters were utilized to construct this consensus tree (from Lachaise *et al.*, 1988). The *melanogaster* subgroup contains three distinct complexes, including the *melanogaster* complex. The *simulans* clade forms an unresolved trichotomy. Its three possible topologies are displayed (a, b, c).

sim = *D. simulans*
sec = *D. sechellia*
mau = *D. mauritiana*


oreno
erecta complex
erecta
teissiera
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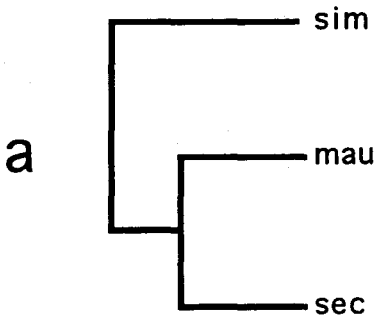
melanogaster
melanogaster complex
simulans
mauritiana
sechellia
simulans clade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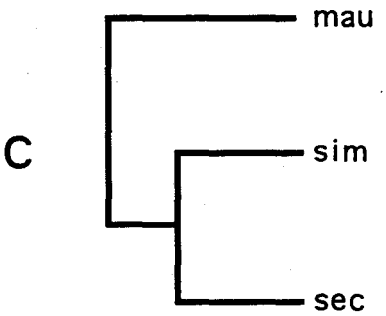
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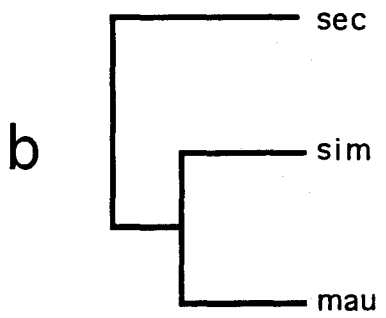
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1.2 Hybridizations between species of the *melanogaster* complex

Interspecific hybridizations between members of the *melanogaster* complex, with the exception of one cross, follow the script of Haldane's rule. When *D. simulans*, *D. sechellia* or *D. mauritiana* females are crossed to *D. melanogaster* males, sterile males result with *no* adult female progeny (Sturtevant, 1920; David *et al.* 1974). This breach of Haldane's rule does not occur in the reciprocal cross as only sterile adult female progeny are produced. However, strains that can rescue the viability of the 'absent' hybrid sex have been discovered from both *D. simulans* and *D. melanogaster* (Watanabe, 1979; Hutter & Ashburner, 1987; Hutter *et al.*, 1990; Sawamura *et al.*, 1993a, b). Recently, certain strains of these two species have been shown to produce *fertile* females when hybridized in one direction of the cross (Davis *et al.*, 1996).

Between all three sibling species, *D. simulans*, *D. sechellia*, and *D. mauritiana*, interspecific hybridizations propagate an invariant production of fertile female and sterile male progeny (Lachaise *et al.*, 1988). In addition, an apparent presence of prezygotic reproductive isolating barriers, in the form of mating discrimination, has created difficulties in producing certain interspecific hybrids in the laboratory (Lee & Watanabe, 1987; Coyne, 1994).

1.3 Spermatogenesis in the *melanogaster* subgroup

The drastic change in cell morphology, from an undifferentiated cell to 64 highly specialized spermatozoa, reveals both the complexity and underlying importance of spermatogenesis. The large majority of information regarding this remarkable process has been observed in *D. melanogaster*. It is believed that spermatogenesis might be well conserved between the different species of *Drosophila*, especially among sibling species, although differences in the number of gonial divisions and the length of time between certain stages of spermatogenesis have been observed between *D. melanogaster* and *D. hydei* (Hennig & Kremer, 1990).

Gametogenesis, in the male, commences soon after fertilization. During gastrulation, several nuclei migrate posteriorly towards the invaginated midgut region. These nuclei interact with the mesoderm of the embryonic gut to form embryonic gonads (Sonnenblick, 1941). Interactions between the rudimentary testes and the independently developing seminal vesicles are important in the proper operation of the reproductive system. By eclosion, the testes are fully formed and are composed of somatic and germline cells (Fuller, 1993).

At the apical end of the testes, a mass of gonial cells is attached to the somatic apical hub (Hardy *et al.*, 1979). Spermatogenesis starts after one of these cells divide and the daughter cell, now called the primary spermatogonial cell, becomes entrapped by a pair of somatically derived cells, the head and tail cyst cells. The primary spermatogonial cell undergoes four rounds of mitoses to create a 16-cell cyst. Cytokinesis is incomplete allowing all spermatids within a sperm bundle to be essentially joined. At this stage, each cell gradually increases in volume and actively transcribes most of the mRNA necessary for sperm

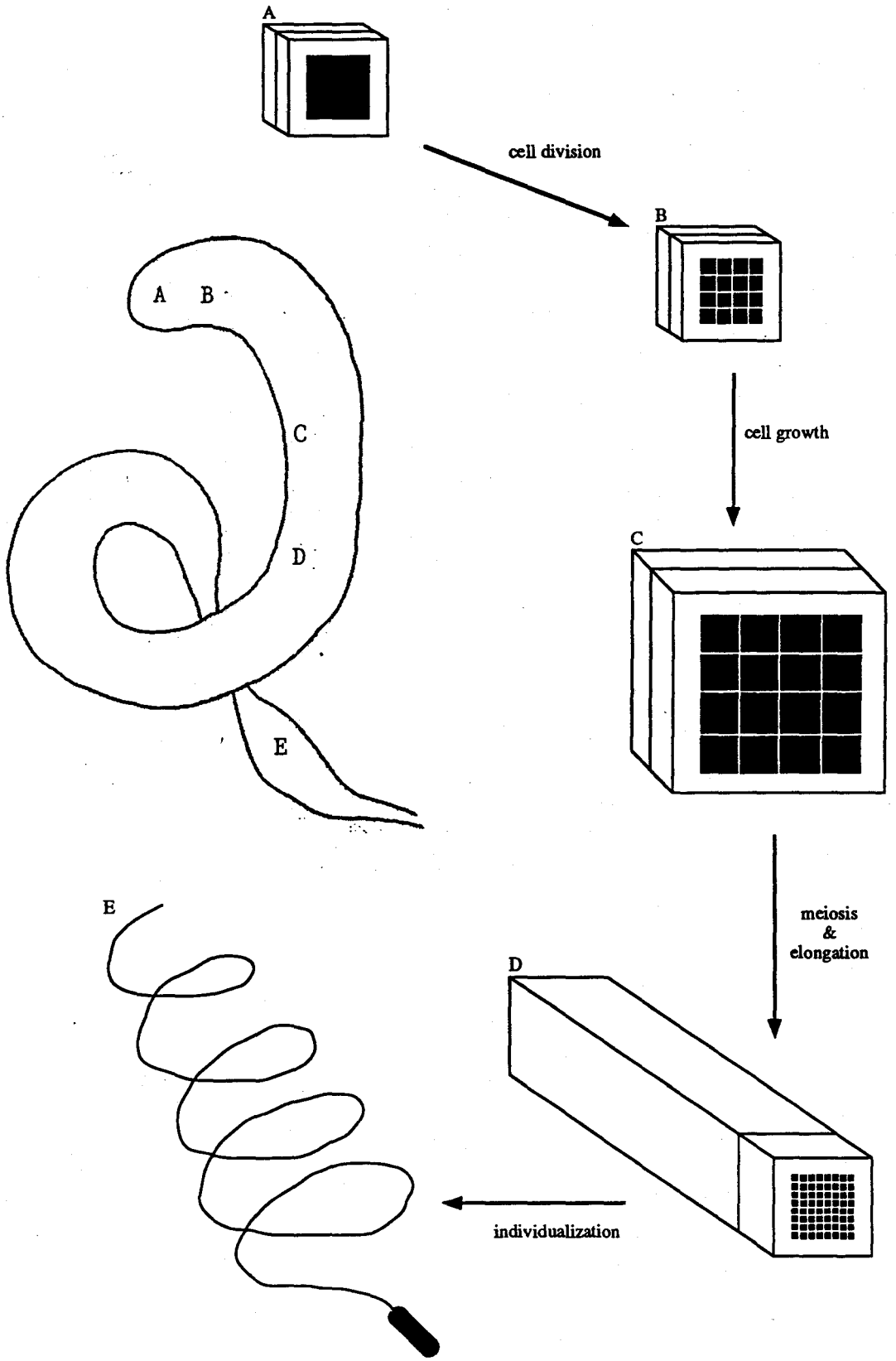
maturation (Olivieri & Olivieri, 1965). Once transcription is completed, the cell will have obtained its maximum size and is known as a primary spermatocyte.

After meiosis, each cyst consists of 64 haploid spermatids engulfed within their head and tail cyst cells. Cytokinesis is still incomplete and cytoplasmic bridges and ring canals are observed to connect adjacent spermatids (Rasmussen, 1973). Eventually, all spermatids of a cyst will elongate, in synchrony, into sperm bundles just under 2 mm long. The extensive differentiation of each spermatid into individualized motile sperm is called spermiogenesis. As the spermatid becomes longer and thinner, three major components within each spermatid must also follow suit. Both the major and minor mitochondrial derivatives, as well as the axoneme (all three make up the axonemal complex) must extend the full length of the sperm tail (Lindsley & Tokuyasu, 1980). At the same time, a dramatic change in nuclei morphology takes place (Tokuyasu, 1974b).

The process of individualization allows each spermatid within a sperm bundle to have full autonomy. Cytoplasmic bridges are severed and excess nucleoplasm and cytoplasm are expelled into a 'waste bag' as each of the spermatids become tightly invested in its own plasma membrane (Tokuyasu *et al.*, 1972a). With the completion of individualization, the sperm bundle begins to coil (Tokuyasu *et al.* 1972b). The head cyst cell becomes entrapped in one of the columnar terminal epithelial cells found at the extreme basal end of the testes. The tail cyst cell, as well as the waste bag, is eventually brought into association with these cells where they are degraded. Finally, after coiling, the mature sperm are released into the testis lumen and find their way into the seminal vesicle (Lindsley & Tokuyasu, 1980).

Figure 1.2 Progression of spermatogenesis in *D. melanogaster*

During the process of spermatogenesis, certain cells undergo a dramatic change of morphology and number while traversing through the testis from the apex to the basal end to finally the seminal vesicle (see Figure 2.3 for description of testes). (A) The diploid primary spermatogonial cell (black square) is engulfed by two somatic cells, the head cyst cell (foreground, surrounding black square) and the tail cyst cell (background). Such cells are located at the apical hub of the testis. After four rounds of mitoses, the resultant cyst (B) will contain sixteen spermatocytes. These spermatocytes will increase in volume while transcription takes place and gradually move basally through the testis. (C) Each spermatocyte, now called primary spermatocytes, proceed through meiosis. Elongation commences near the centre of the testes and equally extends in both directions. (D) Each cyst now contains 64 immature spermatids. Once the head cyst cell reaches the extreme basal end of the testis, it attaches itself to a columnar terminal epithelial cell allowing the sperm bundles to coil and become individualized. (E) After individualization, spermatids are separate from each other and move into the seminal vesicle.


A
cell division
B
A B
C
D
E
cell growth
C
meiosis
&
elongation
D
E
individualization

1.4 Genetic control of normal spermatogenesis

Studies investigating the genetic organization of spermatogenesis *within* species have revealed a number of interesting patterns in *Drosophila*. Such propensities include the quantity of spermatogenic loci found in the genome, the relative amount of male-specific fertility loci compared to female-specific loci, and the types of spermatogenic genes that are most amenable to mutation (see Lindsley & Lifschytz, 1972).

Because of its ultimate importance, loci involved in fertility occupy a large portion of an organism's genome. The genome of *Drosophila* is no exception as there are approximately 15 % as many male sterile mutations as recessive lethals for both the X-chromosomes and the autosomes (Lindsley & Lifschytz, 1972). In another estimate, using P-element mutagenesis, the ratio of male-sterile to lethal mutations was 1:10 (Castrillon *et al.*, 1993.) In *D. melanogaster*, the number of loci that can mutate to lethality has been estimated to be 3750 (Judd *et al.*, 1972). Hence the number of potentially male-sterile loci is in the range of 400 - 600. This quantity is in fact a gross underestimate since the amount of temperature sensitive mutants was not calculated into the sum. If they were, the total amount of loci involved in spermatogenesis may be in the range of 1250-1750 (Lindsley & Lifschytz, 1972).

But spermatogenesis is not the sole program of reproduction. Female fertility, in the programme of oogenesis, is also represented by an appreciably large number of loci. However, in *Drosophila*, the amount of loci affecting female fertility is significantly smaller than the number of male fertility loci. For example, Lindsley and Lifschytz (1972) found that among 413 autosomes containing one or more EMS induced sterile mutations, 231 affected males only, 140 affected females only, and 25 affected both sexes (the remaining 17 were

determined to be double mutants). It must be noted that many of these mutations exhibit such pleiotropic effects as reduced viability (Lifschytz & Hareven, 1977) that may indicate a non-specific fertility effect. Furthermore, the low number of both-sex sterile mutants may indicate that a large amount of differentiation has evolved between the two processes of oogenesis and spermatogenesis.

The phenotypic nature of male-sterile mutants also conveys an interesting trend. Although less than half of the germinal cycle time is involved in spermiogenesis, the majority of genetic male-sterile mutants produce defects in this final spermatogenic stage (Lindsley & Lifschytz, 1972). Other studies, using chemical mutagens (Lifschytz, 1987; Hackstein *et al.*, 1990; Hackstein, 1991) as well as P-element mutagenesis (Castrillon *et al.*, 1993), exhibit a similar predominant range of postmeiotic mutations. Mutations affecting germ-cell proliferation, spermatocyte development and meiotic mechanisms were relatively infrequent.

In addition to the genic instances of sterility, discussed above, sterility may be caused by chromosomal phenomenon. Over 75 % of X-autosomal translocations produce a male-specific sterile phenotype (Lindsley, 1965). Other chromosomal translocations do not have such an effect on male fertility. Changes in the relative timing of condensation and decondensation between the X-chromosome and the autosome have been thought to lead to these abnormalities in spermatogenesis (Lindsley & Lifschytz, 1972; Jablonka & Lamb, 1991) since a translocation will disturb the X-chromosome's precocious inactivation. Again, most of these sterile mutants manifest defects postmeiotically in spermiogenesis (Lindsley & Tokuyasu, 1980).

1.5 Hybrid male sterility

While within species male sterility results from the individual interaction of a mutant locus to other conspecific loci, the epistatic interaction(s) between two distinct and diverged genomes is the cause of hybrid male sterility. Haldane's rule provides the first impression that the incidence of hybrid male sterility may be solely accounted by the genetic constitution of a hybrid individual (i.e. heterogametic vs. homogametic sex chromosomes). The large effect of the X-chromosome in hybrid male sterility, sometimes termed as the "second rule of speciation" (Coyne & Orr, 1989), also suggests a sex chromosomal influence (Dobzhansky, 1936; Orr, 1987; Charlesworth *et al.*, 1987). Although this 'rule' has been argued to be a hemizygous observational bias (since comparisons were made between hemizygous X-linked loci and heterozygous autosomal loci), a number of recent studies have resolved this problem. True *et al.* (1996) generated X-chromosomal P-element introgressions of *D. mauritiana* into a *D. simulans* background that are male sterile, in addition to sterile *homozygous* autosomal segment introgressions. They observed 50 % more hemizygous X-linked steriles than homozygous autosomal male steriles. Accordingly, a number of hypotheses concerning the interaction of various chromosomes with the X-chromosome have been proposed to explain the occurrence of hybrid male sterility in *Drosophila.*

An interaction between the X-chromosome and the autosomes had first been suggested by Haldane (1922) and then by Muller (1940). In this hypothesis, the loss of complementary interactions between X-chromosome and autosomal loci, caused by the gradual divergence of loci in each species, may result in sterility. Another version of the X-autosome interaction was proposed by Dobzhansky (1937b). He believed that each species

has a particular balance of genes on each chromosome that have become different from other species through events such as translocations. Unlike female hybrids, male hybrids are missing parts of the X-chromosome that correspond to the paternal set of autosomes. This genic *imbalance* renders the hybrid sterile. Coyne (1985) disproved this explanation of Haldane's rule (which in fact represents a subset of the X-autosomal interaction hypothesis) with the simple demonstration that a hybrid F_1 female, with both X chromosomes originating from the mother (through an attached-X cross) and heterozygous autosomes, is fully fertile.

Evidence of X-autosomal interactions causing hybrid male sterility has been apparent in many diverse genera of *Drosophila*. This most prevalent form of chromosomal interaction has been observed in such species pairs as *D. pseudoobscura / D. persimilis* (Dobzhansky, 1936), *D. pseudoobscura pseudoobscura / D. pseudoobscura bogotana* (Prakash, 1972), *D. hydei / D. neohydei* (Schafer, 1978), *D. virilis / D. lummei* (Heikkinen & Lumme, 1991) and *D. simulans / D. sechellia* (Zeng & Singh, 1993). An alternative explanation of the over-representation of heterogametic hybrid sterility, involving the X-chromosome, is the X-Y interaction hypothesis (Haldane, 1932). A few instances of this interaction have been documented (Coyne, 1985; Orr, 1987, Hennig, 1977). Such interactions between the X and Y chromosomes, however, may not be common (Johnson *et al.*, 1992; Zeng & Singh, 1993).

Y-autosomal incompatibilities have also been proposed to explain hybrid male sterility. Hybrids between *D. virilis* and *D. lummei* (Heikkinen & Lumme, 1991), as well as *D. mojavensis* and *D. arizonensis* (Vigneault & Zouros, 1986; Pantazidis & Zouros, 1993), manifest such interactions. Consequently, interactions between the sex chromosomes and autosomes have been regarded as most important in the early stages of hybrid male sterility.

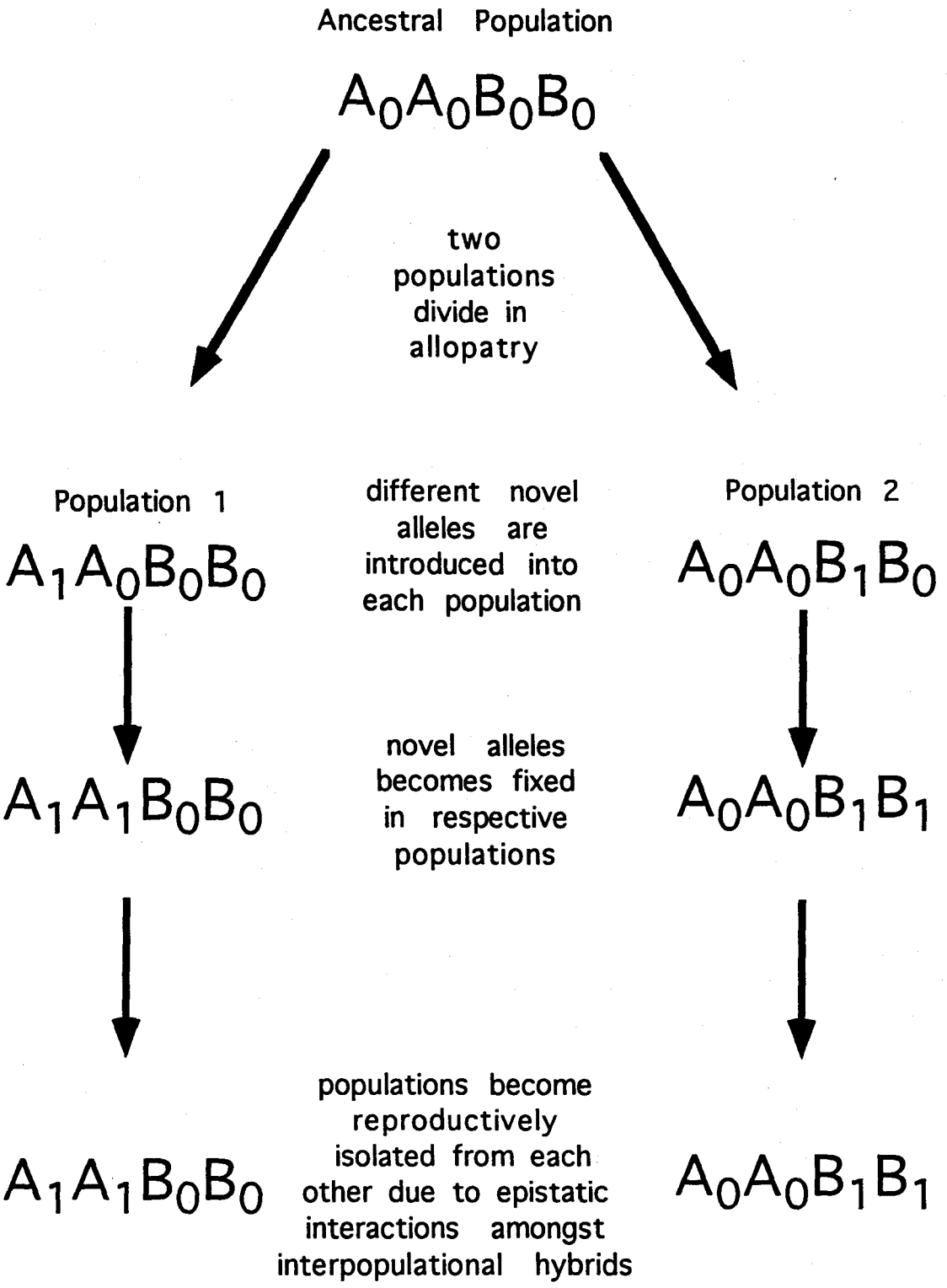
1.6 Models of speciation

While genic interactions between heterospecific chromosomes has been shown to result in hybrid male sterility, the evolution of this reproductive isolation mechanism has historically posed a significant problem. How can a maladaptive trait, such as hybrid sterility, evolve by natural selection, without one of the two parental genotypes passing through an adaptive *valley*? Dobzhansky (1937b) and Muller (1939, 1942) solved this problem by producing genetic models showing that the two diverging populations need not suffer any intermediate loss of fitness (Figure 1.3). In fact, their simple models show that the difficulty of the origin of species can be "reduced to the building up of complimentary genes" (Orr, 1995).

Their two-locus models can be summarized as follows: Two allopatric populations initially have identical genotypes at two loci, $A_0A_0B_0B_0$. In one of the populations, a new A allele, A_1, appears and becomes fixed. $A_1A_0B_0B_0$ and $A_1A_1B_0B_0$ genotypes are perfectly viable and fertile. In the other population, a new B allele, B_1, appears and becomes fixed. $A_0A_0B_1B_0$ and $A_0A_0B_1B_1$ are perfectly viable and fertile as well. Both A_1 and B_1 alleles have never "met" and are potentially incompatible with each other. A resulting hybrid $A_1A_0B_1B_0$ genotype may therefore be sterile. Muller (1942) showed that these two substitutions could occur in both populations, or just one. In addition, the genes involved may be selected for through their pleiotropic effects on other characters, or by the effect of random genetic drift in small populations (Nei, 1976).

Figure 1.3 Two-loci model of reproductive isolation

The Dobzhansky-Muller two-loci model of the evolution of reproductive isolation. Before becoming geographically separated from one another, the two populations are genetically identical. After isolation, two new alleles must independently become fixed. One allele may become fixed in *each* population (as in the figure) or both alleles may become fixed in *one* population. Regardless of the cause, once the second derived allele becomes fixed, an incompatible interaction may result. This potential incompatibility is caused by the fact that the second derived allele has never had the chance to interact with the ancestral allele of the first locus.


Ancestral Population
$A_0A_0B_0B_0$
two populations divide in allopatry
Population 1
$A_1A_0B_0B_0$
different novel alleles are introduced into each population
Population 2
$A_0A_0B_1B_0$
$A_1A_1B_0B_0$
novel alleles becomes fixed in respective populations
$A_0A_0B_1B_1$
$A_1A_1B_0B_0$
populations become reproductively isolated from each other due to epistatic interactions amongst interpopulational hybrids
$A_0A_0B_1B_1$

1.7 Asymmetrical nature of intergenomic interactions

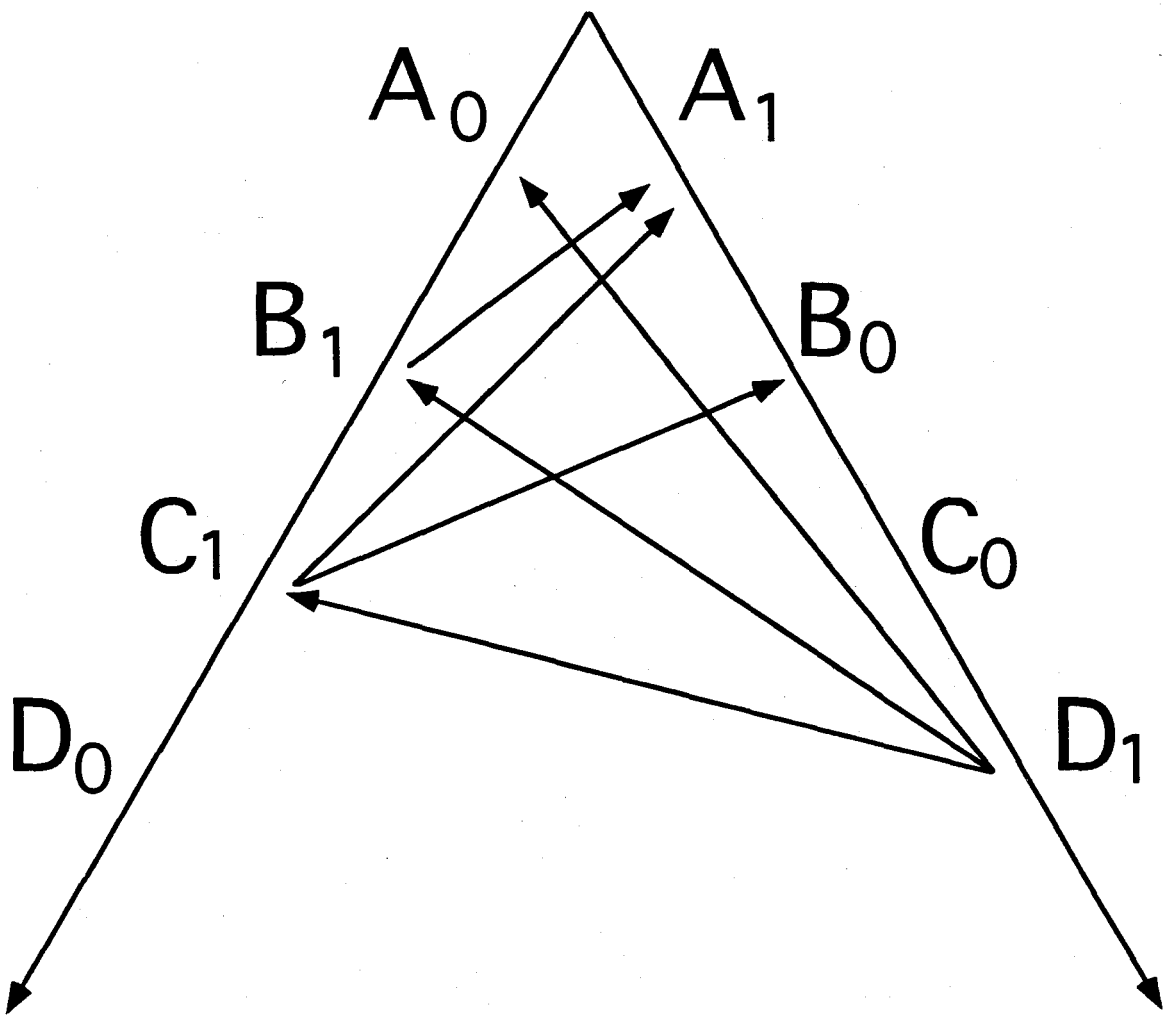
A consequence of the Dobzhansky-Muller two locus speciation model is the invariable presence of genic asymmetry in all hybrid incompatibilities (Muller, 1942). For example, if A_1 has evolved an incompatibility with B_1, the interaction between the alleles A_0 and B_0 will *not* produce an incompatibility. This asymmetry results because the alternative alleles of an incompatible interaction had been represented, in consonance with each other, during intermediate ancestral steps. (Note that such asymmetries do *not* mean that both derived and ancestral alleles of one locus can not produce incompatibilities but that the derived and ancestral alleles of the first locus can not *both* be incompatible with alleles of the second locus.)

Orr (1995) modelled the accumulation of these incompatibilities and noted a number of obvious themes. First, the confirming observation that all genic incompatibilities are asymmetric becomes conspicuously apparent (Figure 1.4). The second theme pertains to the fact that evolutionary derived alleles are involved in more potential incompatible interactions than ancestral alleles. Thirdly, substitutions that occur later in divergence will cause more incompatibilities than those that were fixed at an earlier stage. This increase in incompatibility rate implies "that the strength of reproductive isolation might increase faster than linearly with time" (Orr, 1995).

Empirically, nonreciprocal hybrid male sterility (observational asymmetry) seems to be quite common between closely related species when both reciprocal combinations were assayed for fertility (Bock, 1984). (Reciprocity refers to the existence of two F_1 male genotypic types produced from two interspecific parental crosses; species-A♀ X species-B♂

Figure 1.4 Accumulation of incompatibilities

The increase in the number of incompatible interactions between loci of two populations through time. The ancestral population consists of the alleles, $A_oB_oC_oD_o$. A newly fixed allele is represented by n_1 . The first substitution occurred at locus A, the second at B, the third at C, and the fourth at D. Arrows represent the possible two-locus incompatibilities.


A0
A1
B1
B0
C1
C0
D0
D1

and species-B♀ X species-A♂.) This form of asymmetry is found in situations where hybrid males of one interspecific cross are fertile while the hybrid males of its reciprocal cross are sterile, or whereby homologous chromosomal segment introgressions, amongst reciprocal crosses, manifests a disparity in hybrid male fertility (Wu & Beckenbach, 1983). Such observational asymmetries, however, are a direct result of genic asymmetries and comply with two-locus models of reproductive isolation only when one of the loci is situated on the sex chromosomes. Hence, the presence or absence of nonreciprocal hybrid male sterility may be predicted in such models by specifying the specific chromosome that each affected locus is carried on (Zouros, 1986; Zeng & Singh, 1993). For example, the interaction of a newly derived sex-linked locus from species A with a derived autosomal locus from species B, will bring about the presence of observational asymmetry in the form of nonreciprocal hybrid male sterility. Other chromosomal associations, that involve two loci on the same chromosome type (such as the interaction between two autosomal loci), will result in symmetrical sterility interactions, in the form of reciprocal hybrid male sterility. However, since most of the evidence has favoured the incompatible interactions between loci on one of the two sex chromosomes and loci found on the autosomes, the presence of nonreciprocal hybrid male sterility may be considered a standard fixture in the early stages of hybrid male sterility (see **1.5**).

The presence of reciprocal F_1 male sterility, is apparent among many interspecific hybridizations. Such interactions are likely to occur after the species have separated long enough allowing other loci to interact deleteriously (Wu & Beckenbach, 1983). Since the number of fertility loci that may create incompatibilities in the hybrid is quite large, the

progression of nonreciprocal to reciprocal hybrid male sterility may be rapid. Such observational symmetries would not be caused by a *specific* symmetrical genic interaction (between two alleles of a homologous locus). These symmetrical interactions should not appear at an early stage because the probability of such a symmetrical interaction is quite small, as it represents the square of the probability of *one* substitution at that particular locus (Orr, 1995). In addition, different pairs of species, even within the same phylogenetic cluster, would be expected to employ different sets of interacting genes in the sterile hybrid male (Wu *et al.*, 1992).

1.8 Maternal and cytoplasmic effects on hybrid male sterility

In addition to the nuclear genic interactions that are known to cause incompatibilities in the hybrid, other factors, inherited through the egg's cytoplasm, have been thought to influence hybrid male sterility (Dobzhansky & Sturtevant, 1935; Dobzhansky, 1937b). These factors, which may have the potential to interact deleteriously with the hybrid's genome, can be categorized under two types, maternal effects and cytoplasmic determinants (or inheritance). Maternal effects consists of all gene products that arise from the maternal nuclear genome and are transmitted though the egg's cytoplasm to the zygote. Cytoplasmic determinants are factors produced from autonomously reproducing entities found in the cytoplasm, and transmitted via the cytoplasm through the maternal lineage to the hybrid zygote.

The distinction between maternal effect and cytoplasmic inheritance is important in regards to the classical model of speciation (Dobzhansky, 1970). According to this

conventional view of species formation, reproductive isolation results from ordinary mutations at common loci. Although maternal effects are consistent with the classical view, cytoplasmic determinants represent a departure from the model. Thus, transmitted factors such as transposable elements whose dysgenic effects depend on the egg's cytotype (Bingham *et al.*, 1982), as well as endosymbionts such as streptococcal L-forms (Somerson *et al.*, 1984; Hoffmann, 1986) precipitate an exception to the classical view of speciation (Goulielmos & Zouros, 1995).

A number of studies, utilizing a variety of methods, have attempted to procure evidence on the incompatibility of the hybrid's genome with maternal effect factors. Orr (1989) has claimed that the difference between F_1 sterile male hybrids derived from the species, *D. pseudoobscura pseudoobscura* and *D. pseudoobscura bogotana*, and F_2 males of the 'same' genotypic constitution can be explained by a maternal effect (Dobzhansky, 1974). In most studies, however, the disentanglement of factors originating from the X-chromosome and factors stemming from the cytoplasm are impossible. This problem can be overcome with the use of a cross employing an attached-X line (see Figure 2.1, in materials & methods). Such crosses with attached-X females produce patriclinous sons that inherit the X-chromosome from their father and both the Y-chromosome and cytoplasm from their mother (Lindsley & Zimm, 1980). Using recombinant F_2 males of *D. simulans* and *D. mauritiana*, Davis *et al.* (1994) has shown that maternal effects, as well as cytoplasmic determinants, do not seem to be factors in the sterility of these hybrid males.

The influence of cytoplasmic determinants on hybrid male sterility, at least in *Drosophila*, has been also demonstrated to lack experimental support. Zeng & Singh (1993)

have shown, through recurrent backcrossing, that introgressed cytoplasms in pure species backgrounds have no effect on hybrid male sterility between the three species of the *simulans* clade. Using an 'incompatibility analysis', Goulielmos & Zouros (1995) have revealed a lack of evidence for cytoplasmic inheritance on the sterility of male hybrids between *D. arizonae* and *D. mojavensis*.

1.9 Objectives of the study

Spermatogenesis is a complex and important developmental process. Differences in this mechanism between closely related species, however subtle, effect an expensive toll on the hybrids in the form of fertility loss. Are these differences apparent in comparative screens of testes structure? Do these traits have high or low variances? What is the effect of intraspecific variation on hybrid male sterility? Answers to such questions may primarily assist us in understanding the onset of hybrid male sterility.

The cytological characterization of hybrid male sterility permits a greater understanding into the formation of species by revealing the *nature* of the interactions involved early in this process. Through the observations of a range of developmental anomalies at the F_1 level in different interspecific hybridizations, we may be able to roughly assess the kinds of genes involved in the production of incompatibilities in the form of hybrid male sterility.

Obviously, finely-tuned genetic dissections can not be made simply by observing the F_1 hybrid, but by observing the *complete* interaction of two divergent genomes, accurate profiles of developmental patterns in male hybrids may be ascertained. The presence of

asymmetries in the sterility phenotype, between reciprocal crosses, represents one such pattern. Other patterns may relate to the phylogeny of the species utilized.

And finally, the fundamental question of the role of cytoplasmic factors in hybrid male sterility will be assessed. Can the sterility of the heterogametic sex be the result of interspecific interactions between the cytoplasm and the chromosomes?

Materials and Methods

2.1 *Drosophila* stocks

Two different strains from each of *Drosophila simulans*, *D. sechellia*, and *D. mauritiana* were utilized in this study. In addition, an attached-X line (Figure 2.1) from *D. simulans* was employed, as well as a Peruvian strain of *D. melanogaster*. A description of their origins and sources is listed in Table 2.1.

2.2 *Drosophila* stock culture

All *Drosophila* stocks were cultured in 250 ml glass jars with foam plugs on approximately 25 ml of standard banana medium (Table 2.3). These stocks were then maintained at 22 - 23 °C in an incubator under a 12 hour dark/light cycle.

2.3 *Drosophila* hybridizations

All hybridizations (within strain, between strain, and between species) took place in 35 ml glass vials with foam plugs and approximately 5 ml of standard banana medium. Both virgin male and female flies were collected from stock cultures within a five day period prior to mating. Flies were anaesthetized using low levels of $CO_{2\,(g)}$ in order to separate the sexes. In all matings, 10 females were crossed to 10 males (again in the presence of low levels of

Table 2.1 List of *Drosophila* stocks utilized in study

Species	Origin	Strain	Source
D. melanogaster	Peru	2419	Bowling Green S.R.C.[a]
D. simulans	Colombia	0251.2	Bloomington D.S.C.[b]
D. simulans	South Africa	914	Bloomington D.S.C.
D. simulans	attached X-line (see Figure 2.1)	2119	Bloomington D.S.C.
D. mauritiana	Mauritius, derived from isofemale line	LG-24	Dr. Jean David
D. mauritiana	Mauritius, derived from isofemale line	2252	Bloomington D.S.C.
D. sechellia	Seychelles, derived from isofemale line	3590	Dr. Jean David
D. sechellia	Seychelles, derived from isofemale line	3588	Bloomington D.S.C.

[a] from the National *Drosophila* Species Resource Center at Bowling Green State University, Ohio

[b] from the *Drosophila* Stock Center in Bloomington, Indiana

Figure 2.1 Description of the attached-X line

The attached-X line (Bloomington stock number *2119*) utilized in this study possessed the particular constitution, *yvmgf*^{2} / C(1) RM *yw*. The compound-X chromosome is shown in white. Only two of the four sex chromosomal combinations are viable. In males produced from attached-X lines, the X chromosome is inherited from the father while the Y chromosome is maternally inherited. The cytoplasm is, as usual, inherited maternally. Female flies can be distinguished from their male counterparts by their *white* eyes. Males possess a multiply marked X-chromosome that includes the following; *yellow*, *vermillion*, *miniature*, *garnet*, and *forked*.

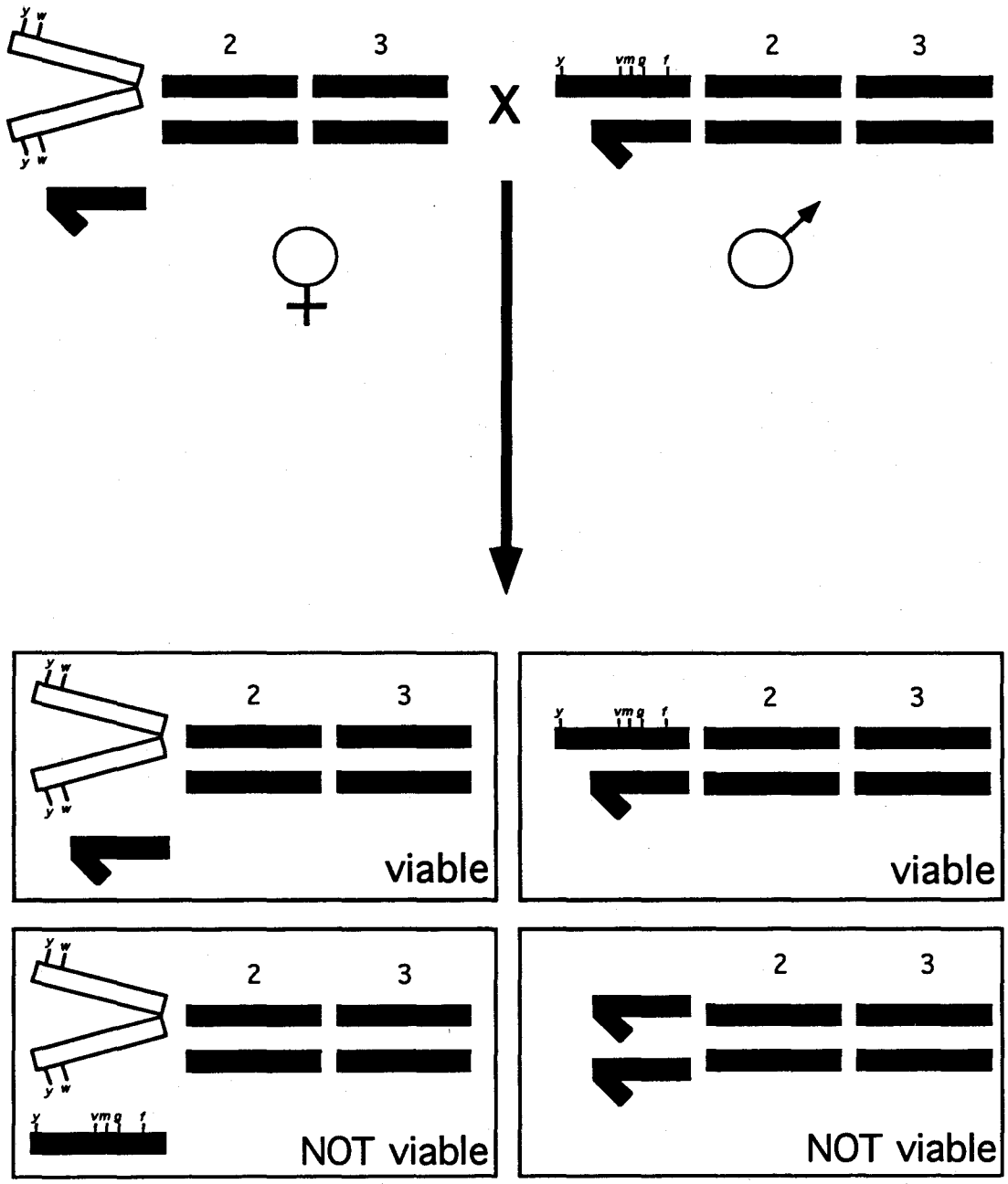

y w
y w
2
3
X
y
vm g
f
2
3
viable
viable
NOT viable
NOT viable

Table 2.2 Source of chemicals

Chemical Name	Abbreviation	Source[a]
Acetic Acid$_{(aq)}$ (Glacial)	CH_3COOH	1
Albumin (nuclease-free bovine serum)	-----	12
Calcium Chloride (anhydrous)	$CaCl_2$	2
Carbon Dioxide$_{(g)}$	CO_2	3
Chloroform	$CHCl_3$	1
1,2-Dichloroethane	-----	6
Diglycidyl Ether of Polypropylene Glycol	DER 736	4
Dimethylaminoethanol	DMAE	4
Ficoll	-----	6
Formvar	-----	4
D-Glucose (anhydrous)	$C_6H_{12}O_6$	2
Gluteraldehyde	-----	4
Hydrochloric Acid$_{(aq)}$	HCl	5
p-Hydroxy-Benzoic Acid Methyl Ester	$C_8H_8O_3$	6
Lead Citrate	$Pb_3(C_6H_5O_7)_2$-$3H_20$	7
Nonenyl Succinnic Anhydride (EM grade)	NSA	4
Orcein (natural red 28), synthetic	-----	6

Table 2.2 (cont'd)

Chemical Name	Abbreviation	Source[a]
Osmium Tetroxide	OsO_4	4
Paraformaldehyde	$(CH_2O)_n$	8
Permount	-----	5
Polyvinylpyrrolidone 360	-----	6
Potassium Chloride	KCl	9
Potassium Hydroxide	KOH	2
Propylene Oxide	C_3H_6O	2
Schneider's liquid medium	-----	13
Sodium Cacodylate	-----	4
Sodium Chloride	NaCl	9
Sodium Citrate	-----	10
Sodium Phosphate, Monobasic (anhydrous)	NaH_2P0_4	6
Sodium Phosphate, Dibasic	Na_2HP0_4	9
Tris (hydroxymethyl)nitromethane	Tris	2
Uranyl acetate	-----	4
Vinylcyclohexene dioxide	ERL 4206	4
Yeast	-----	11

Table 2.2 (cont'd)

a

1 - Caledon Laboratories Ltd., Georgetown, Ontario

2 - BDH Chemicals Inc., Toronto, Ontario

3 - Liquid Air, Hamilton, Ontario

4 - Marivac Ltd., Halifax, Nova Scotia

5 - Fisher Scientific, Nepean, Ontario

6 - Sigma Chemical Co., St. Louis, Missouri

7 - K & K Laboratories, Plainview, New York

8 - BDH Laboratory Supplies, Poole, England

9 - Fisher Scientific, Fair Lawn, New Jersey

10 - J.T. Baker Inc., Phillipsburg, New Jersey

11 - Fleischmann's Yeast, LaSalle, Quebec

12 - Boehringer Mannheim, Laval, Quebec

13 - Gibco BRL Products, Gaithersburg, Maryland

Table 2.3 Standard banana medium for *Drosophila*

water	1.8 l
agar	20 g
bananas	2 medium sized
corn syrup	2 tbsp
brewer's yeast	60 g

Dissolve the agar in boiling water. Mix the remaining ingredients in a blender and add to the boiling agar solution. Permit the mixture to boil and then allow it to cool. When the media has been cooled to 45 °C, add 36 ml of tegosept (10 g of methyl p-hydroxy benzoate in 100 ml 95% ethanol). After careful mixing, pour the media into the appropriate containers (250 ml jar or 35 ml vial) with the aid of a 50 cc syringe.

$CO_{2\ (g)}$) with the exception of one cross. In the interspecific cross between *D. sechellia* females and *D. simulans*, between 15 and 20 males were mated to 10 females. In all crosses, parents were repeatedly transferred to fresh vials every 5 - 7 days for a period of four weeks.

Crossed flies and their progeny were subjected to the same timed-light and temperature conditions as flies from the original stock cultures (see **2.2**).

2.3.1 Crosses used to investigate cytoplasmic influences

Hybridizations between pure species lines allow for the production, and subsequent cytological analysis, of F_1 male-steriles. The utilization of an attached-X line (*D. simulans*-2119) in this study further grants us the power to search for possible factors, in hybrid male sterility, that may be maternally transmitted through the cytoplasm. However, the presence of differences in sterility between hybrids that are genotypically identical, except for the origin of their cytoplasm, does not conclusively demonstrate that a cytoplasmically inherited factor is the source of these differences.

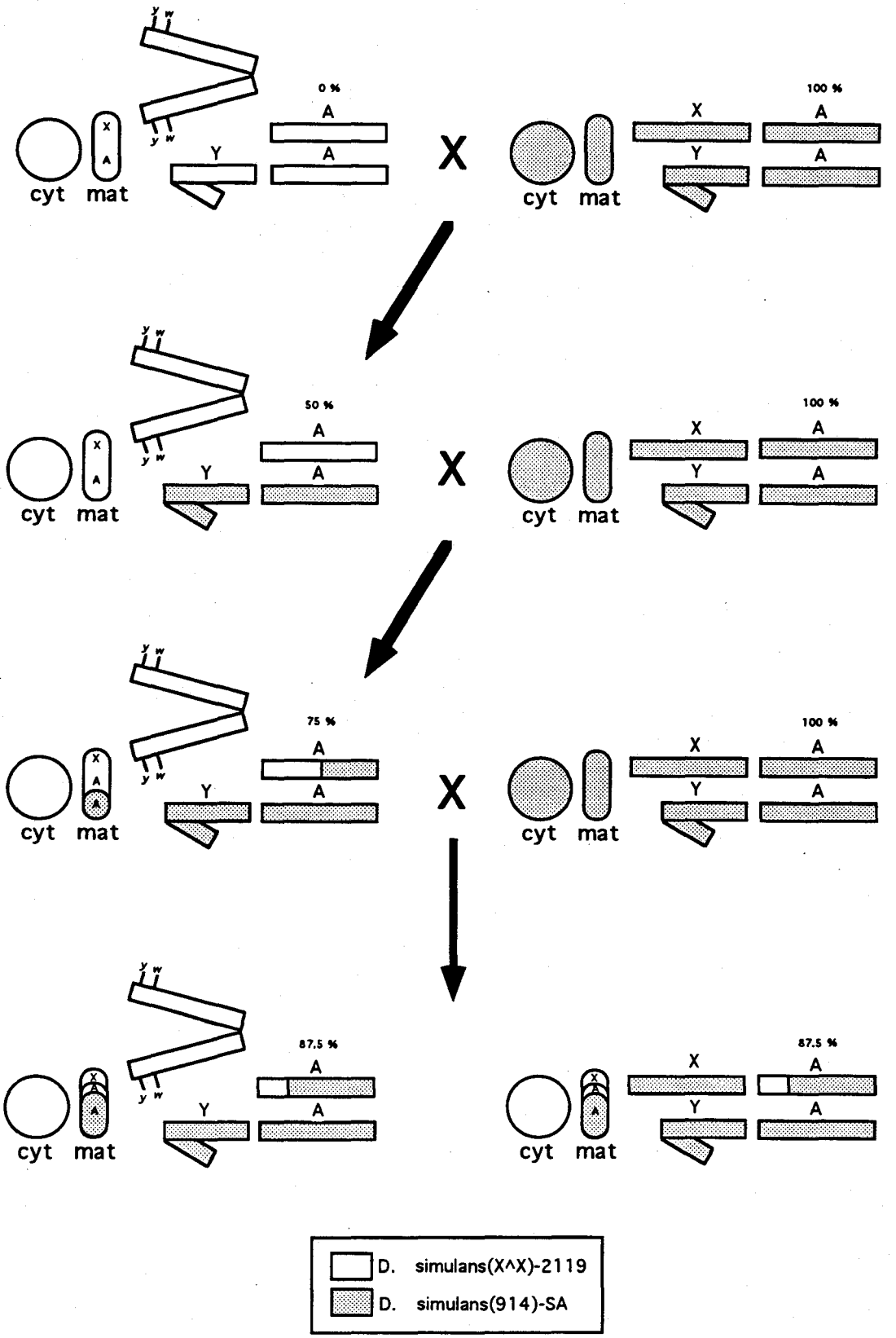
In order to test this possibility, a 'new' attached-X line was created. By backcrossing virgin female flies of an attached-X stock to males of another strain of *D. simulans*, the attached-X's genetic background can be gradually replaced by that of the other strain. The resulting line will eventually harbour a different genetic background but still retain its original attached-X transmission characteristics. The cross illustrated in Figure 2.2 produces such a line. Attached-X female progeny from *D. simulans*X^X (2119) were backcrossed to males of *D. simulans*, South Africa (914), for two generations.

Figure 2.2 Cross used to resolve effects of the cytoplasm

This crossing scheme was employed in order to replace the genetic background of the existing attached-X line with that of another. Virgin females from a *D. simulans* attached-X line (2119) were initially crossed to South African (914) *D. simulans* males. The female progeny produced from this cross were then backcrossed to males of this South African (SA) strain for a total of two generations. The resultant stock will possess free X and Y chromosomes that are completely South African in origin, autosomes and maternal effects whose genetic constitution are largely South African in origin, and compound-X chromosomes and cytoplasm that have fully retained their original *2119* genetic identity. The 'X', 'Y', and 'A' refer to the X, Y, and autosomal chromosomes, respectively.

cyt = cytoplasm
mat = maternal effects

The percentages listed above the autosomes pertain to the relative amount of autosomal content originating from the South African (914) strain. These percentages are calculated on the basis of free recombination and random association of chromosomes. The autosomal component of the maternal effect that stems from the South African strain is equal in proportion to the autosomal content of this strain in the mother.


y w
y w
X
A
Y
A
0 %
cyt
mat
X
100 %
50 %
75 %
87.5 %
D. simulans(X^X)-2119
D. simulans(914)-SA

2.4 Whole mount testes analysis

The presence and coinciding behaviour of spermatid bundles and individualized sperm may be efficiently assessed by the observation of unruptured testes/seminal vesicles as a whole through different levels of magnification in both the testes and the seminal vesicle.

2.4.1 Tissue dissection

Using a Zeiss dissecting microscope, the reproductive tract was retrieved from live flies within 16 hours of eclosion while submerged under a drop of Hennig's testes' buffer (Table 2.4). Since the dissections were done at night (corresponding to the dark phase of the flies' environment), the majority of flies would be close to 16 hours old since most flies eclose soon after the dark-to-light transition in the morning (Ashburner, 1989). A pair of Dumont (#5) forceps was used to grasp the anterior end of the abdomen (surrounding the posterior process of the genital arch) in place while another pair of forceps was utilized to cleanly extract the reproductive tract by posteriorly drawing out the eighth abdominal segment. Testes and seminal vesicles were then isolated from the rest of the reproductive tract tissues. A Corning 22 mm^2 coverslip was gently placed on top of the testes preparation.

Table 2.4 Hennig's testes buffer

1 M KCl	183 ml
1 M NaCl	47 ml
1 M Tris-HCl (pH 6.8)	10 ml
distilled water	to one litre

Sterilize solution in glass bottle by autoclaving.

2.4.2 Microscopy analysis

The testes were then promptly observed, under brightfield, phase-contrast, and Nomarski optics using a Zeiss Axioplan microscope, for the presence of various spermatogenic landmarks. The apical end (or apex) of the testis was defined as the area at the closed-ended tip of the testis, one tenth the total testis' length (see Figure 2.3). The midtestes region was approximated to be the area (one tenth the total length of the testis) in the exact middle of the testis. The testis' basal region, like the apex, was defined as the area traversing the final tenth of the testes. Terminal epithelial cells occur in the extreme basal region of the testis where it attaches to the seminal vesicle. These columnar shaped cells can be seen in Figure 3.12. Photographs were taken with the aid of a 35 mm film cassette Mot attachment using TMAX 100 film.

Using this procedure, the presence of sperm bundles in different areas of the testes could easily be quantified. Care was taken to ensure that each sperm bundle was only counted once. If too many sperm bundles were present at a certain location, the maximum number of sperm bundles that could clearly be resolved was listed in conjunction with a greater-than symbol, '>'. As well, this procedure allows for an evaluation to be made on the presence of individualized spermatids in the seminal vesicle. Such an assessment was most easily produced by observing the testes and seminal vesicles under Nomarski optics (also known as Differential Interference Contrast) found in the Zeiss Axioplan microscope.

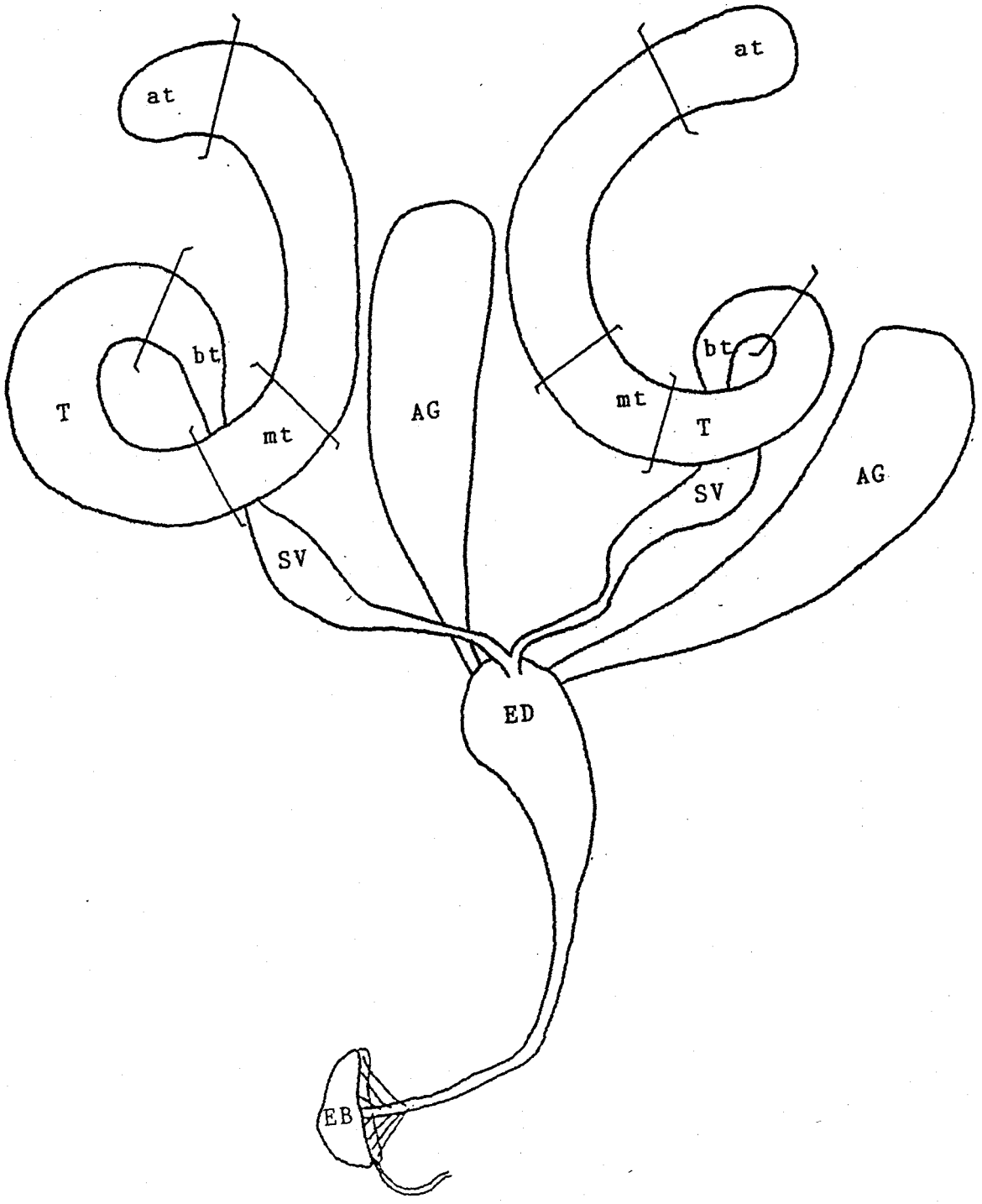
Figure 2.3 Testes description

A schematic illustration of the male reproduction tract is employed to explain the various terms used in this study.

T	=	testis
SV	=	seminal vesicle
AG	=	accessory gland (paragonium)
ED	=	anterior ejaculatory duct
EB	=	ejaculatory bulb

Within each testes, boundaries of three areas (denoted in lower-case) are outlined. The apex was defined as the area in the closed-ended tip of the testis in which the average width had not been obtained (in the extreme tenth of the total testis area). The midtestis and basal regions were defined similarly for their respective areas.

at	=	apical end of testis (apex)
mt	=	middle of testis (midtestis)
bt	=	basal region (end) of testis


at
at
bt
bt
T
T
mt
mt
AG
AG
SV
SV
ED
EB

2.5 Orcein staining of spermatid heads

To trace the behaviour of sperm bundle head nuclei in hybrids that have a *post*meiotic arrest, an orcein stain of the testes was performed. This procedure is a combination of the Lifschytz & Hareven (1977) and Szabad *et al.* (1994) protocols.

2.5.1 Tissue dissection

Using a Zeiss dissection microscope, testes derived from three day old virgin males (66-74 hours) were dissected in a hypotonic (0.075 M) KCl solution using the dissection procedure as described above (**2.4.1**). While being aged, flies were kept in standard vial conditions at low density for the entire duration of their adult life, until testes fixation.

2.5.2 Staining and slide preparation

Dissected testes were allowed to stand in 0.075 M KCl solution for ten minutes followed by a five minute fixation in a 6:3:1 ethanol:chloroform:acetic acid solution. Testes were then immersed in absolute ethanol for ten minutes. A hydration series followed whereby testes were forced through descending concentrations of alcohol (95%, 90%, 70%, 50%) to distilled water for a period of ten minutes each.

Testes were then subjected to 1 N HCl for thirty minutes. Staining with 2 % orcein in 45 % acetic acid (Ashburner, 1989) took place for five minutes. The aceto-orcein stain was then replaced by 45 % acetic acid. Testes were then placed on a slide, covered with a coverslip, and promptly observed using a Zeiss Axioscope.

2.6 Embedding of testes

In order to view testes morphology at a more refined level, testes were infiltrated with a plastic resin which consequently allowed the tissue to be subjected to ultrastructural analyses through thick (light microscopy) and thin (electron microscopy) testis cross-sections.

2.6.1 Tissue dissection

Again using the dissection procedure as described above (**2.4.1**), testes were extracted from three day old virgin male flies while submerged in Schneider's liquid medium. These flies were collected within eight hours of eclosion, and were aged in standard vial conditions at low density from their time of collection for sixty-six hours. Hence, each adult testis is between 66 and 74 hours old. In order to maintain testis sample independence, usually one testes was randomly dissected from each individual. If a pair of testes was retrieved, these testes would be registered as a pair when embedded in their respective blocks.

2.6.2 Primary and post-fixatives

Testes were bathed in the primary fixative (Table 2.5). Immediately, testes were fixed in fresh primary fixative on ice for sixty minutes. The fixative was removed and testes were then washed in 0.1 M cacodylate buffer (Table 2.5) six times, for ten minutes each. Testes were then postfixed in 1.0 % osmium tetroxide in 0.1 M cacodylate buffer for one hour on ice. The osmium tetroxide solution was removed and the testes were again washed

Table 2.5 Primary fixative for embedded tissue

0.2 M cacodylate buffer (pH 7.4)		10 ml
To make approximately 100 ml of 0.2 M buffer:		
1) Dissolve 4.2 g sodium cacodylate into 100 ml water		
2) Add 5.4 ml 0.2 M HCl. Stir. Adjust pH to 7.4 with 0.2 M HCl.		
4 % paraformaldehyde		5 ml
To make 100 ml of 4 % paraformaldehyde solution:		
1) Combine 4 g paraformaldehyde and 0.1 g $NaOH_{(s)}$		
2) Add distilled water to 85 ml and stir to dissolve		
3) Add the following:		
	1 % calcium $chloride_{(aq)}$ 0.5 ml	
	glucose 1.2 g	
	1 M NaH_2PO_4 4.0 ml	
	1 M Na_2HPO_4 6.0 ml	
4) Check pH. Add distilled water to 100 ml.		
25 % glutaraldehyde		2 ml
distilled water		3 ml
	TOTAL =	20 ml

This fixative should be freshly made within 2-3 days of fixation and stored at 4 °C.

in 0.1 M cacodylate buffer six times, for ten minutes each. Testes were then dehydrated in an ascending series of ethanols from 50% to 70% to 90% for a period of ten minutes each. Dehydration was completed following two ten minute washes in 95% ethanol and three ten minute washes in 100% ethanol.

2.6.3 Tissue infiltration with Spurr's resin

Testes were twice transferred to propylene oxide, for a period of ten minutes each. Incubations in increasing amounts of Spurr's resin (Table 2.6) in capped glass test tubes ensued. At first, testes were placed in a 2:1 mixture of propylene oxide to Spurr's resin for thirty minutes. This was followed by testes transfer into a 1:1 mixture of propylene oxide to Spurr's for another thirty minutes. Testes were then incubated in the last mixture at 4 °C for twelve hours. Propylene oxide mixtures were replaced with 100 % Spurr's resin and placed on a shaker for 24 hours. Infiltrated testes were finally placed in embedding molds and baked at 65 °C for eight hours.

Hardened blocks were then collected, sorted, and stored in appropriately labelled glass vials until needed for sectioning. Such embedded blocks can last indefinitely.

Table 2.6 Spurr's resin

Vinylcyclohexene dioxide$_{(l)}$ (ERL 4206)	10.0 g
Diglycidyl ether of polypropylene Glycol$_{(l)}$ (DER 736)	6.0 g
Nonenyl succinic anhydride$_{(l)}$ (NSA)	26.0 g
Dimethylaminoethanol$_{(l)}$ (DMAE)	0.4 g

Gently mix all of the ingredients, except for the DMAE, using a magnetic stirrer for a period of fifteen minutes. Add the DMAE and mix for an additional 20 minutes. Draw the mixture into 10 cc syringes and store at -20 °C until needed.

2.7 Thick sectioning of testes

An RMC MT-7 microtome was used to section embedded tissue. Sections, two microns in thickness, were pared into a waterfilled commercial plastic boat that was attached to a premade glass knife. Once between ten and twenty-five sections had accumulated on top of the surface of water in the boat, a platinum loop was employed to transfer these sections to a subbed glass slide (see Table 2.7). The origins of these sections, from each testes, were diagrammatically recorded. After the whole testes was sectioned, the glass slide was placed on a 65 °C hotplate for one to two minutes in order to allow the sections to spread and adhere to the glass properly. Sections were then promptly stained with 1 % toluidine blue in borax at 65 °C for 10-15 seconds.

2.7.1 Analysis of thick sections under the microscope

For analysis of the thick sections under a compound microscope, the section-ladened slides were covered by a thin layer of Permount and then immediately covered by a 22 X 40 (or 50) mm^2 Corning coverslip. After drying, slides were observed through various magnifications with a Zeiss Axioscope under brightfield. Pictures were taken with TMAX 100 film.

The cross-section of the midtestes region was used to quantify the number of sperm bundles present. Unless stated otherwise, five testis blocks were randomly sampled to be sectioned for each testis type (i.e. within strain or between species crosses). The midtestes region was defined as before (**2.4.2**) and was naturally established by relating each area of sections on the slide to the diagram ascribed to each testis sectioned (**2.7**).

Table 2.7 Subbed slides

To make 2400 ml of the SSC-Denhart solution:

20X SSC (sodium chloride/sodium citrate)	360 ml
1 % polyvinylpyrrolidone 360	48 ml
1 % Ficoll	48 ml
1 % nuclease-free bovine serum albumin	48 ml
distilled water	1896 ml

Incubate clean slides for 2.5 hours at 65 °C in SSC-Denhart solution. Rinse the slides in distilled water for five seconds and immediately fix in 3:1 ethanol:acetic acid fixative for twenty minutes at room temperature. Allow the slides to air-dry. Store slides at 4°C until needed.

2.8 Thin sectioning of testes

In order to properly observe testis sections with the electron microscope, the sections had to be between 60 to 90 nm thick. The same microtome setup, as that described for thick sections (**2.7**), was employed for thin sections of the midtestis region.

2.8.1 Collection of thin sections

After a ribbon of sections had formed in the boat, a xylene saturated wooden stick was waved over the sections in order to expand them. Sections were then carefully positioned by a single-haired brush and placed onto a formvar coated (Table 2.8) copper slot grid. This delicate task was accomplished by slipping the grid beneath the surface of the water and angling it so that the ribbon would fall across the clear formvar face. Grids would then be carefully blotted dry by filter paper before being safely stored in grid storage boxes.

2.8.2 Post-staining of thin sections

Before use on the electron microscope, grids were separately post-stained with both uranyl acetate and lead citrate (as described below). The grids were placed in a petri dish, covered with a 1:1 mixture of uranyl acetate saturated in H_20 and ethanol for thirty minutes (in the dark) and then rinsed in distilled water. Grids were then placed in a petri dish containing potassium hydroxide pellets, stained in lead citrate (Reynolds, 1963) for five minutes, rinsed in carbon dioxide free water, and finally dried. The post-stained grids were stored in labelled grid storage boxes.

Table 2.8 Formvar coating grids

For a 2 % solution of formvar:

formvar (anhydrous)	2 g
di-chloroethane (anhydrous)	100 ml

Keep formvar solution dry and tightly sealed at room temperature until use.

Submerge clean, dust-free microscope slide into formvar solution. Allow slide to dry vertically in formvar vapour. Stand slide vertically for one minute. Score edges with a razor blade. Breathe on one side of slide. Slowly immerse slide into dustfree water at a 45 ° angle and allow formvar film to freely float. Carefully drop copper grids one by one onto floating film. Lay a fresh piece of parafilm on top of the grids and film. Flip and place into a covered petri dish until needed.

2.9 Electron microscopy

Ultrastructural defects in spermiogenesis were analyzed with the use of a JEOL 1200 EX II transmission electron microscope under a large range of magnifications. Images were photographed on Kodak 4489 film and printed on Ilford print paper.

2.10 Statistical analyses

A nested anova was utilized to test the species differences as well as the strain (within species) differences in sperm bundle number (page 55). A nested anova (hierarchical anova) is a single classification anova whereby a subordinate classification level is nested within a higher level of classification. The variance ratio, F, is the ratio of the variances (Mean Square) of the two classification levels and is used to calculate significance. Nested anova, as well as other statistical calculations, employed SAS JMP (version 2.0.2 for the Macintosh).

In the comparison between midtestes sperm bundle number, two tests were employed that took into account the small sample sizes. A Mann-Whitney U-test is a nonparametric test that is analogous to the parametric t-test. In this test, the difference between population *medians* is evaluated by ranking observational values, selecting the larger U-statistic of the two samples, $U_1 = n_1n_2 + 0.5n_2 (n_2 + 1) - R_2$, where R_n is the rank sum for the other sample and n_i is the size of each sample, and then performing a significance test (Campbell, 1989). A Kruskal-Wallis analysis of variance of ranks is similar to a Mann-Whitney test except that more than two samples may be used. The H-statistic computed distributes approximately as a chi-square distribution; $H=[12 / n(n+1)] \Sigma R_i^2/n_i - 3(n+1)$, where n is the total sample size, n_i the size of each respective sample and R_i is the rank sum of each sample (Campbell, 1989).

Results

3.1 Differences between the species

The four species of the *melanogaster* complex were assessed for a number of traits that are diagnostically involved in the process of spermatogenesis. The presence of spermatozoa in the seminal vesicle, as well as coiled sperm bundles in the basal region of the testes, was scored in all of the males produced from intraspecific (within and between strain) crosses (Figure 3.1). Two other fertility characters, found in Table 3.1, were quantified in males produced from intraspecific crosses and pure strain crosses. The first character ascribed the number of sperm bundles found at the apex of the testes in males observed within 16 hours of eclosion while the second trait revealed the number of sperm bundles present at the midtestes region of males that are 66-72 hours old posteclosion. These two characters were measured by two different methods; the former character is quantified through whole mount observations under Nomarski optics while the latter is measured through brightfield observations of cross-sections of embedded testes. Midtestes sperm bundle counts represent a reliable quantification of fertility for a number of reasons. The maximum number of sperm bundles is found in the midtestes region since this site represents the origin of sperm bundle elongation (Tokuyasu *et al.*, 1972b). As well, the midtestes region does not contain coiled or 'looped' sperm bundles which may affect the accuracy of the total sperm bundle count.

Table 3.1 reveals that much of the observed differences in traits can be attributed to

differences between strains within species (nested ANOVA; F=4.051; d.f.=4,32; p<0.01). This highly significant result contrasted the non-significant differences found between species (nested ANOVA; F=2.111; d.f.=3,4; p=0.242). The large strain-to-strain difference is quite evident in Figure 3.2 describing the number of sperm bundles in the midtestes region for the different strains utilized in the study.

The large intraspecific differences and the unexpectedly large variation in spermatogenic traits do not permit these four species to be ranked according to such fertility traits. There may, however, be an apparent difference in sperm bundle content between *D. sechellia* and the other species of the complex. In testes less than 16 hours old posteclosion, lower sperm bundle counts are observed at the apical end of the testes in *D. sechellia*, relative to other species (Table 3.1). As well, in midtestis' cross-sections from males that are three days old posteclosion, strains of *D. sechellia* reveal smaller amounts of pre-elongation spermatid cysts than other species strains (Table 3.1). In addition, the number of midtestes sperm bundles seem to be smaller in *D. sechellia.* However, in other species (i.e. *D. mauritiana* and *D. simulans*), there is a large range in the means of this trait between strains.

Differences in sperm bundle count appear more pronounced in very young testes. Such a significant difference, between *D. simulans* (Colombia) and *D. sechellia* (3590), can be seen in Figure 3.5 in midtestis cross-sections that are less than two hours old posteclosion (Mann-Whitney; $n_1=6$; $n_2=3$; $U_1=0$; $p < 0.05$).

Table 3.1

Intraspecific comparison of testes structure and spermatogenic characters among species of the *melanogaster* complex

Intraspecific cross	Number of sperm bundles at apex of testis[a]	Number of sperm bundles in midtestis cross-section[b]	Number of pre-elongation spermatid cysts in midtestis cross-section[b]	Number of premeiotic cysts in midtestis cross-section[b]
(females X males)	mean (SD, n)	mean (SD, n)	mean (SD, n)	mean (SD, n)
D. simulans				
sim (South Africa)	3.92 (3.4, 26)	54.8 (11.1, 5)	2.0 (1.2, 5)	2.4 (2.3, 5)
sim (S. Africa) X sim (Colombia)	4.15 (3.7, 34)	-	-	-
sim (Colombia) X sim (S. Africa)	3.10 (3.2, 30)	-	-	-
sim (Colombia)	7.58 (3.6, 90)	34.4 (2.7, 5)	2.0 (0.6, 5)	2.2 (0.7, 5)
sim (Colombia) X sim (2119)	6.52 (3.9, 70)	-	-	-
sim (X^X) X sim (Colombia)	4.38 (3.7, 42)	-	-	-
sim (X^X)	1.54 (2.0, 44)	37.4 (13.8, 5)	2.0 (0.7, 5)	2.8 (1.9, 5)
D. sechellia				
sec (3590)	0.0 (0, 52)	26.0 (6.9, 5)	0.6 (1.3, 5)	3.0 (1.6, 5)
sec (3590) X sec (3588)	0.18 (0.7, 28)	-	-	-
sec (3588) X sec (3590)	0.14 (0.5, 28)	-	-	-
sec (3588)	0.74 (1.1, 34)	28.0 (4.0, 5)	0.2 (0.8, 5)	2.2 (1.5, 5)
D. mauritiana				
mau (LG24)	4.78 (3.5, 32)	33.4 (10.3, 5)	1.2 (0.8, 5)	3.8 (1.1, 5)
mau (LG24) X mau (2252)	2.53 (2.1, 34)	-	-	-
mau (2252) X mau (LG24)	3.28 (2.2. 20)	-	-	-
mau (2252)	1.96 (1.8, 26)	21.0 (2.9, 5)	1.0 (0.7, 5)	3.0 (1.0, 5)
D. melanogaster				
mel (Peru)	7.26 (3.2, 34)	34.4 (9.2, 5)	1.6 (0.8, 5)	2.6 (1.3, 5)

[a] Apical testes data were obtained through Nomarski optics on whole mount preparations observed within 16 hours posteclosion.

[b] Data obtained from five independently embedded testis per line that were aged 66-72 hours after eclosion.

X^X refers to the attached-X line *D. simulans*

Figure 3.1 Normal spermatogenesis in the pure species

The normal progression of spermatogenesis in members of the *simulans* clade. Observations were obtained using Nomarski optics at two different magnifications. (A) Whole mount testis view from the midtestis region to the testicular duct of *D. simulans* (Colombia) revealing large numbers of sperm bundles (small arrows). Coiling is evident at the basal region (large arrows) and is fully apparent at the testis' basal (bt) region in (B) just prior to the spermatids becoming individualized and displaced into the seminal vesicle (SV). (C) Dislodged sperm bundles are released from a ruptured testis of *D. sechellia* (3590). Such sperm bundles contain 64 spermatids syncytially packed within a cyst. (D) In the seminal vesicle of *D. mauritiana* (LG-24), spermatids have become separated from each other and are individually coiled. Scale bar (A-B); 100 μm. Scale bar (C-D); 20 μm.

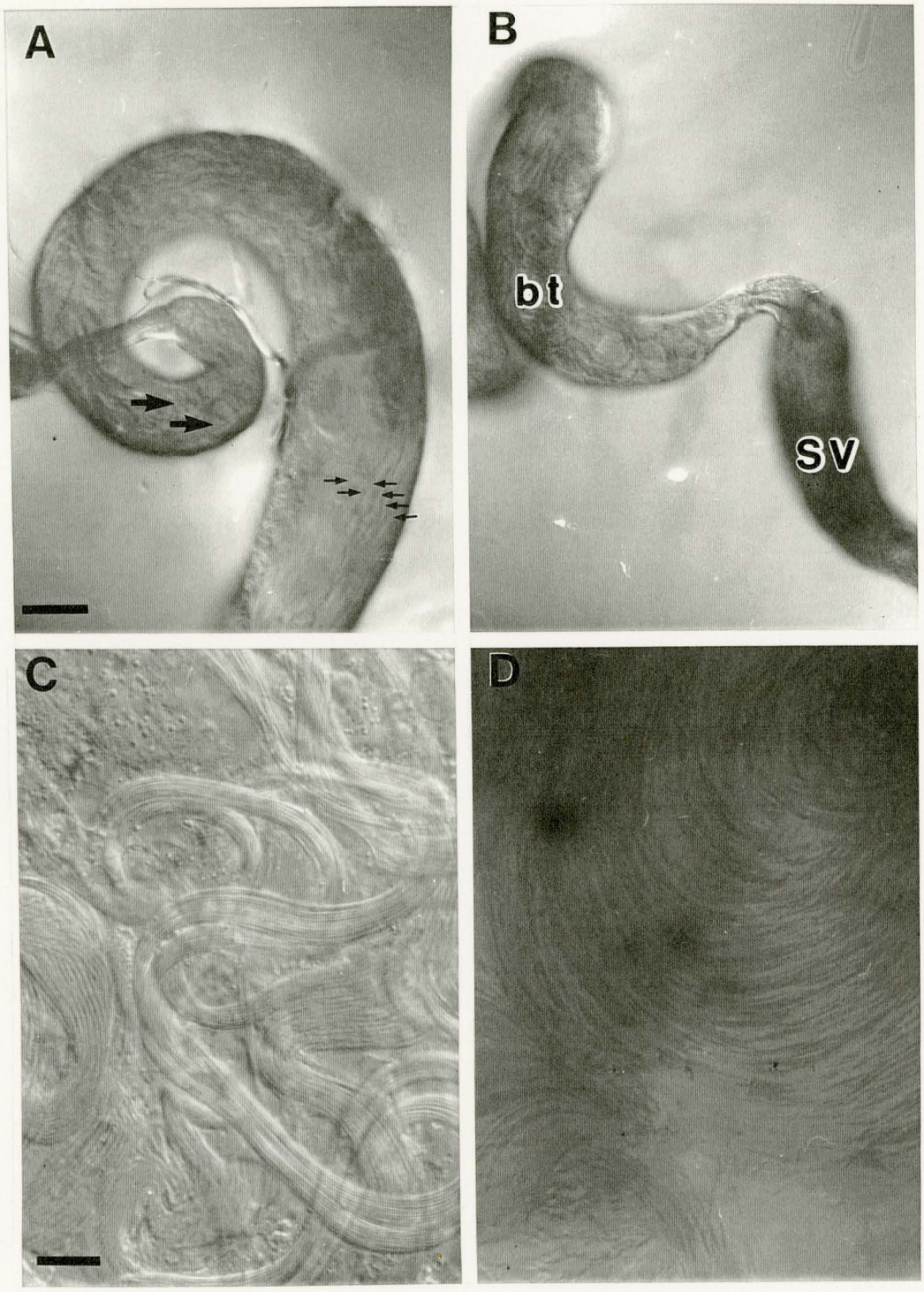
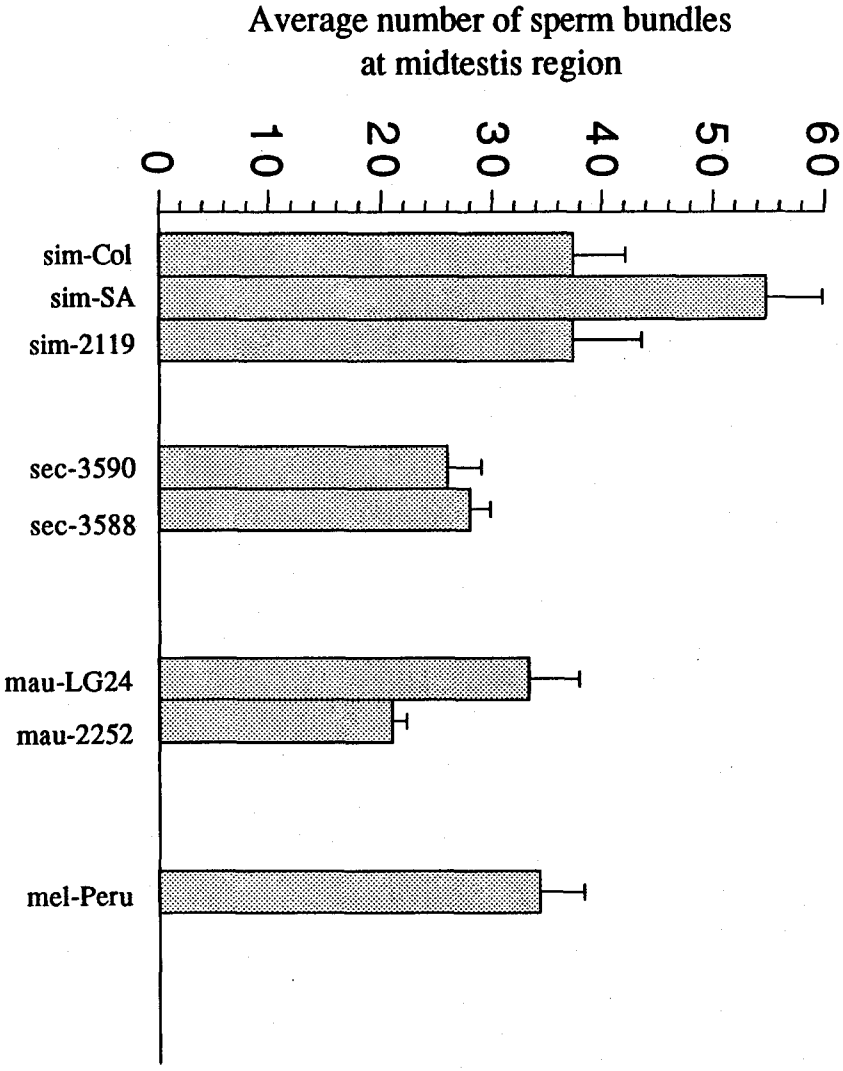

A
B
bt
SV
C
D

Figure 3.2 Amount of sperm bundles in different strains

The average number of mature sperm bundles was assessed for the various strains used in this study. Sperm bundles were counted at the midtestes level in embedded testes that were 66-72 hours old posteclosion. Five randomly sampled testes were evaluated for each line. The standard error of the mean for each strain is represented by the error bars.

D. simulans:	sim-Col	(0251.2, Colombia)
	sim-SA	(914, South Africa)
	sim-2119	(2119, attached-X line)
D. sechellia:	sec-3590	(3590)
	sec-3588	(3588)
D. mauritiana:	mau-LG24	(LG24)
	mau-2252	(2252)
D. melanogaster:	mel-Peru	(2419, Peru)


Average number of sperm bundles
at midtestis region
0
10
20
30
40
50
60
sim-Col
sim-SA
sim-2119
sec-3590
sec-3588
mau-LG24
mau-2252
mel-Peru

3.2 Differences between the hybrids

A schematic diagram (Figure 3.3) summarizes the various products of *inter*specific hybridization, among the *simulans* clade, employed in this study.

3.2.1 Hybrids between *D. simulans* and *D. sechellia*

The three types of F_1 hybrid genotypes, produced from this species pair, could be placed into two distinct classes of spermatogenic defects, postmeiotic and premeiotic (Table 3.2 and Figure 3.4). The presence of sperm bundles (postmeiotic) in both reciprocal crosses of *D. simulans* and *D. sechellia* contrasted the aspermic (premeiotic) nature of testes development in the interspecific crosses involving the attached-X line (*D. simulans*-2119). Both whole mount testes surveys (within 16 hours posteclosion) and cross-sections (66-72 hours posteclosion) confirmed this large difference.

Differences also existed between the two hybrids with postmeiotic abnormalities. Although, the quantities of midtestes sperm bundles, from the hybrids of the two reciprocal crosses of wildtype *D. simulans* and *D. sechellia*, were not significantly different from each other (Mann-Whitney; $n_1=n_2=5; U_1=12$; $p=0.5$), other differences were observed. In the whole mount analysis, there was a complete absence of apical sperm bundles in hybrids of *D. sechellia* (female) X *D. simulans* (male). As well, the sperm bundles of the midtestes regions (testes less than 16 hours old posteclosion) were observed to be larger and more diffuse (especially on the perimeter of the testis lumen) and were not as conspicuously distinct as those of its reciprocal cross. By plotting the number of midtestes level sperm bundles through time, another difference in spermatogenic progression is exhibited (see Figure 3.5). After

three days, the amount of mature sperm bundles with a regular spermatid matrix decreased to zero and much debris was observed in the testis. Such a change in sperm bundle profile did not take place in *D. simulans* (female) X *D. sechellia* (male) hybrids. In fact, an increase in sperm bundle number through time took place in the latter hybrid indicating sperm bundle development through time.

The differences in the degree of sterility between the attached-X line cross and the cross, *D. sechellia* (female) X *D. simulans* (male), are consistent with a cytoplasmic effect since the specific origin of each chromosome is identical in both cases (Figure 3.3). However, an alternative explanation is that this difference in sterility may be due to differences between the two strains of *D. simulans* (attached-X(*2119*) vs. Colombia) utilized in each respective cross.

To reduce the effect on *pre*meiotic sterility caused by the presence of *D. simulans-2119* autosomes, this line was backcrossed to another *D. simulans* line (South Africa) for two generations (see Materials and Methods, Figure 2.2). Females of this novel line, still containing the attached-X chromosome, were then crossed to *D. sechellia* (3590) males. Next, males (2-4 days posteclosion) were embedded and sectioned at the midtestes regions. The results are summarized in Table 3.3. Out of twelve independently sampled testes cross-sections, ten contained postmeiotic arrests in spermatogenesis, corresponding to the same class of postmeiotic sterility as its genotypic equivalent, *D. sechellia* (female) X *D. simulans* (male). Two cross-sections did not seem to contain any postmeiotic cysts at all. However, these two cross-section profiles offered ambiguous results as they could not be placed being more similar to a *D. simulans-X^X* (female) X *D. sechellia* (male) hybrid (which are aspermic

Figure 3.3 Summary of hybrids' genetic constitutions and origins

The products of interspecific hybridizations, amongst the *simulans* clade, are summarized. The three sibling species produce six F_1 hybrids plus an additional two F_1 hybrids that originate from a *D. simulans* attached-X line (2119) cross (found in boxes).

sim = *D. simulans*
sec = *D. sechellia*
mau = *D. mauritiana*

cyt = cytoplasm
X = X chromosome
Y = Y chromosome
A = autosomes

Genomic symbols (ie. cyt, X, Y, and A) are identified on the pure species' genotypic schematics, found at the vertices of the triangle. Crosses producing the hybrid genotypes are as follows;

(1a) sim(X^X)♀ X sec♂ (1b) sim♀ X sec♂ (1c) sec♀ X sim♂

(2a) sim(X^X)♀ X mau♂ (2b) sim♀ X mau♂ (2c) mau♀ X sim♂

(3a) mau♀ X sec♂ (3b) sec♀ X mau♂.

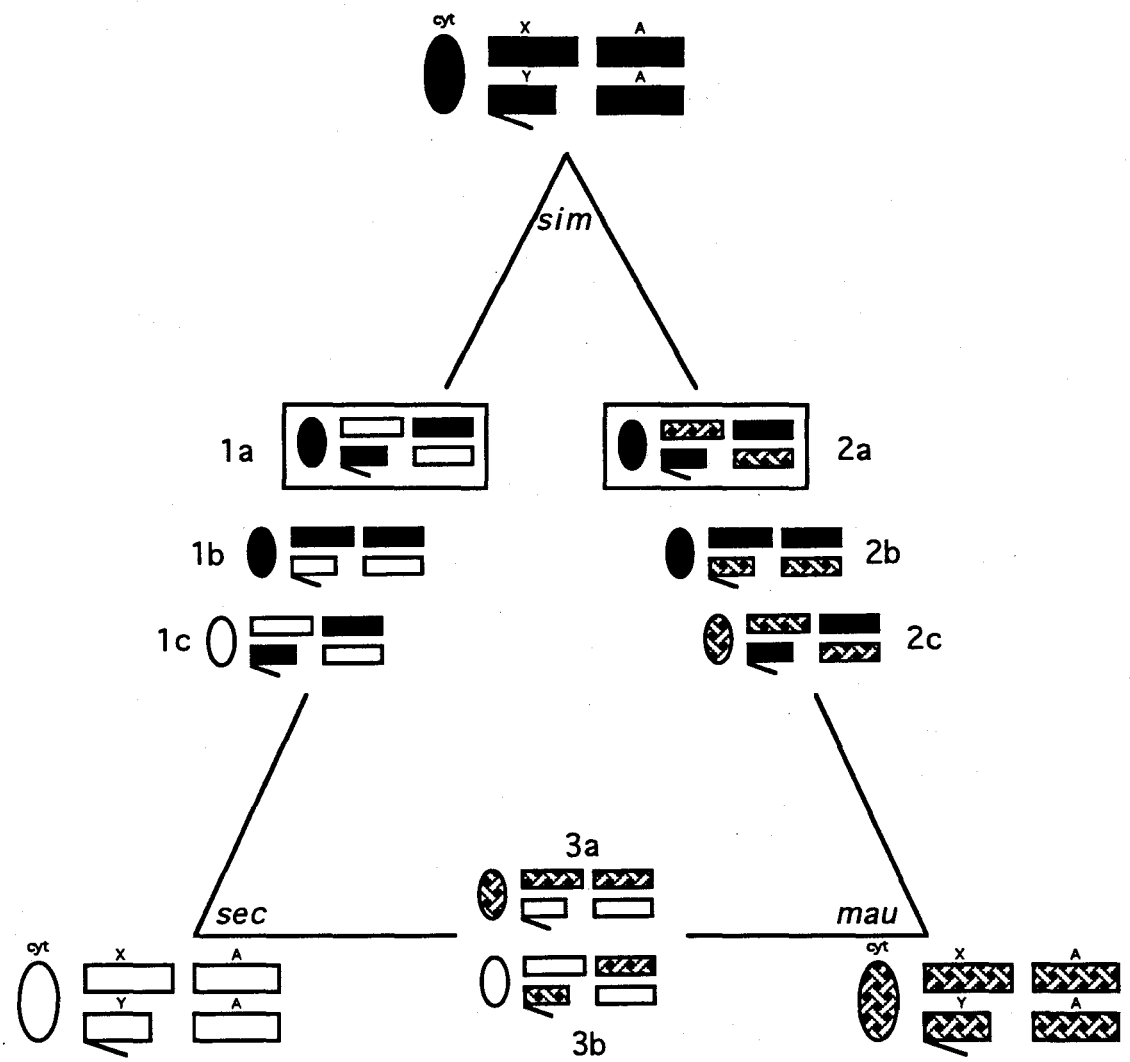

cyt
X
Y
A
A
sim
1a
2a
1b
2b
1c
2c
3a
sec
mau
3b

Table 3.2

Spermatogenic characteristics in interspecific F_1 hybrids of *D. simulans and D. sechellia*

Species Crosses (females X males)	Number of testes examined n	Number of sperm bundles at apex of testis mean (SD)	Presence of elongated sperm bundles at midtestis region	Number of sperm bundles at midtestis region mean (SD)	Number of sperm bundles at midtestis cross-section[a] mean (SD, n)	Number of pre-elongation spermatid cysts at midtestis[a] mean (SD, n)	Number of premeiotic cysts at midtestis[a] mean (SD, n)	Presence of elongated cysts at testis' base	Coiled sperm bundles at testis' base	Sperm bundles at terminal epithelial cell region	Sperm presence in seminal vesicle
D. simulans X D. sechellia											
sim (Colombia) X sec (3590)	140	0.18 (0.7)	yes	>12	30.0 (2.3, 5)	2.4 (0.5, 5)	2.4 (0.7, 5)	yes	no	no	no
sim (Colombia) X sec (3588)	24	0.29 (0.7)	yes	>12	-	-	-	yes	no	no	no
sim (South Africa) X sec (3590)	16	0.31 (0.6)	yes	>12	-	-	-	yes	no	no	no
sim (South Africa) X sec (3588)	18	0.50 (1.0)	yes	>12	-	-	-	yes	no	no	no
D. sechellia X D. simulans											
sec (3590) X sim (Colombia)	78	0.0	yes	can't resolve	31.2 (13.0, 5)	4.0 (2.1, 5)	2.8 (1.1, 5)	yes	no	no	no
sec (3590) X sim (South Africa)	20	0.0	yes	can't resolve	-	-	-	yes	no	no	no
sec (3588) X sim (Colombia)	36	0.0	yes	can't resolve	-	-	-	yes	no	no	no
sec (3588) X sim (South Africa)	22	0.0	yes	can't resolve	-	-	-	yes	no	no	no
D. simulans(X^X) X D. sechellia											
sim (X^X-2119) X sec (3590)	128	0.0	no	0.0	0.0 (0, 5)	0.0 (0, 5)	12.6 (4.0, 5)	small amts	no	no	no
sim (X^X-2119) X sec (3588)	26	0.0	no	0.0	-	-	-	small amts	no	no	no

[a] Data obtained from five independently embedded testes per line (66-72 hours post-eclosion).

All other data was collected from observations of whole-mounted testes under Nomarski optics.

Figure 3.4 Testes of *sim/sec* hybrids

Whole-mounted testes observed under Nomarski optics (left) and midtestes cross-sections (right) of F_1 hybrid testes between *D. simulans* and *D. sechellia*. (A) *D. simulans* (female) X *D. sechellia* (male) hybrid testes display very conspicuous sperm bundles (arrowheads) from the midtestes (MT) region to the testis' base. No coiling at the basal region is apparent. (B) The cross-section reveals ample sperm bundles of all sizes. A range of cyst conditions are present from ones with variable spermatid sizes (curved arrow) to cysts with a seemingly regular spermatid matrix (straight arrows). (C) *D. sechellia* (female) X *D. simulans* (male) hybrids also contain sperm bundles at both MT and basal regions (arrowheads) but these are not as distinct as those found in the *D. simulans* (female) X *D. sechellia* (male) hybrid. (D) However, the cross-section reveals that there are abundant sperm bundles present with distinct spermatids (arrows). Some cysts can be seen with irregular spermatid morphologies (curved arrow). In fact, the presence of large pre-elongated spermatid cysts (arrowheads) may have hidden the sperm bundles in this region. (E) *D. simulans(X^X)* (female) X *D.sechellia* (male) hybrids demonstrate a lack of sperm bundles at the MT region but exhibit diffuse, elongated bundles at the basal area (arrowheads). (F) Sections reveal these cyst 'bundles' (arrowheads) to contain less than 16 cells each. All rightside panels are to the same scale bar as A, 100 µm. All leftside panels are to the same scale bar as B, 20 µm.

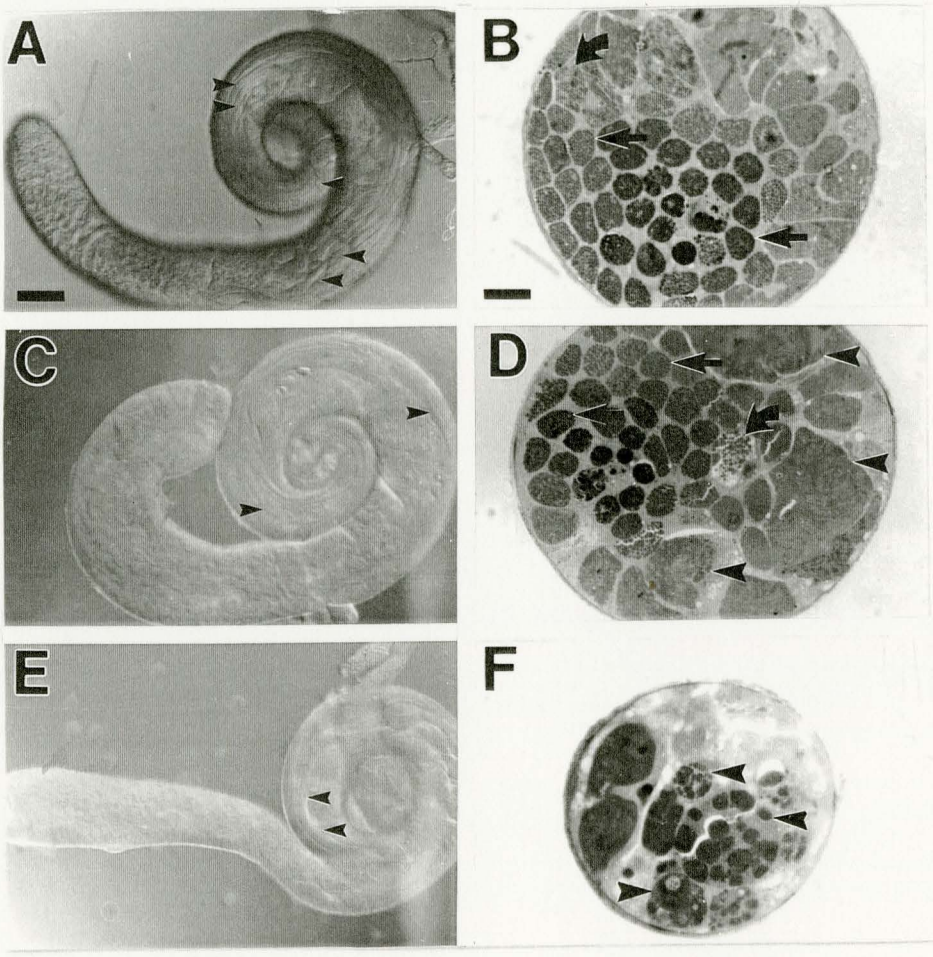
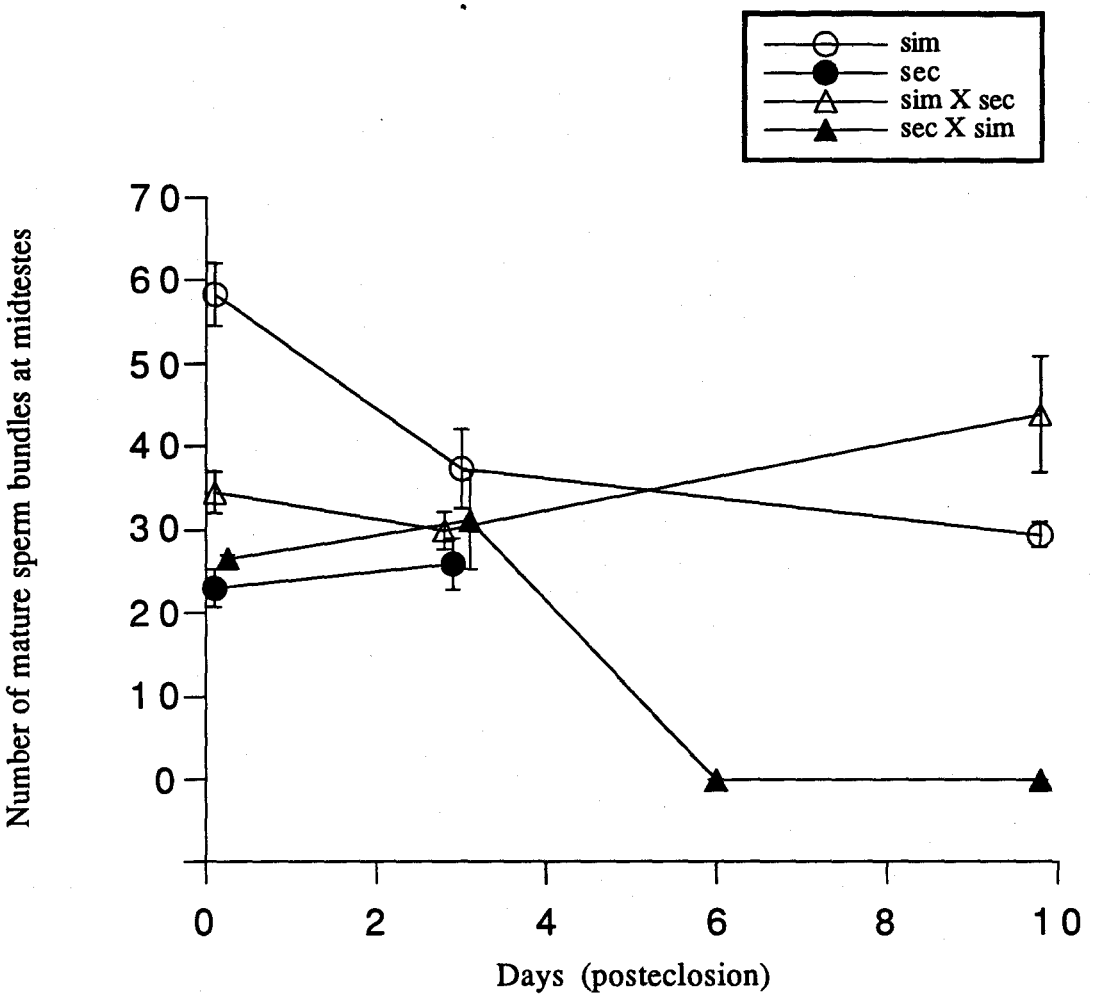

A
B
C
D
E
F

Figure 3.5 Amount of sperm bundles through time in *sim/sec* hybrids

The number of sperm bundles at the midtestes region are assessed at different times after eclosion in *D. simulans* / *D. sechellia* combinations. Errors bars represent the standard error of the mean. The following lines/hybrids (the first species listed represents the female parent), the times that they were sampled (posteclosion), and their respective sample numbers are as follows:

< 2 hrs	*D. simulans* (Colombia)	n=6
	D. sechellia (3590)	n=3
	D. simulans (Colombia) X *D. sechellia* (3590)	n=1
	D. sechellia (3590) X *D. simulans* (Colombia)	n=2
66-72 hrs	*D. simulans* (Colombia)	n=5
	D. sechellia (3590)	n=5
	D. simulans (Colombia) X *D. sechellia* (3590)	n=5
	D. sechellia (3590) X *D. simulans* (Colombia)	n=5
138-144 hrs	*D. sechellia* (3590) X *D. simulans* (Colombia)	n=2
232-240 hrs	*D. simulans* (Colombia)	n=2
	D. simulans (Colombia) X *D. sechellia* (3590)	n=2
	D. sechellia (3590) X *D. simulans* (Colombia)	n=4


sim
sec
sim X sec
sec X sim
Number of mature sperm bundles at midtestes
70
60
50
40
30
20
10
0
0
2
4
6
8
10
Days (posteclosion)

from the time of eclosion) or a degenerating *D. sechellia* (female) X *D. simulans* (male) hybrid (which become aspermic after three days posteclosion) since the testes used in this analysis were between 2-4 days old posteclosion. A comparison was then made on the sterility phenotype of the ten 'reverted' postmeiotic steriles to the postmeiotic steriles of the *sim/sec* reciprocal hybrids. In Figure 3.4, it is shown that the average number of midtestes sperm bundles was significantly less than either of the two hybrids of the reciprocal crosses (Kruskal-Wallis; X^2_2=6.29; p=0.0430).

Table 3.3

Midtestes analysis of interspecific F_1 hybrids between *D. simulans* and *D. sechellia*
Effect of different genetic background on male sterility of *D. simulans* (X^X) X *D. sechellia* hybrids

Interspecific Crosses	Number of testes assayed	Number of testes with postmeiotic defects	Percentage of testes with postmeiotic defects	Number of sperm bundles in midtestis X-section	Number of pre-elongation spermatid cysts in midtestis cross-section	Number of premeiotic cysts in midtestis cross-section
(females X males)	n		%	mean (SD)	mean (SD)	mean (SD)
sim (X^X-2119)[a] X sec	5	0	0	0	0	13 (2.3)
sim (X^X-2119/914)[b] X sec	12	10	83	21 (3.6)[c]	3.5 (1.0)[c]	2.6 (0.6)[c]

[a] sim (X^X-2119) refers to the original attached-X line (2119)

[b] sim (X^X-2119/914) refers to the backcrossed attached-X line (see fig 2.2) with a different *D. simulans* background

[c] only testis with postmeiotic cysts were tallied

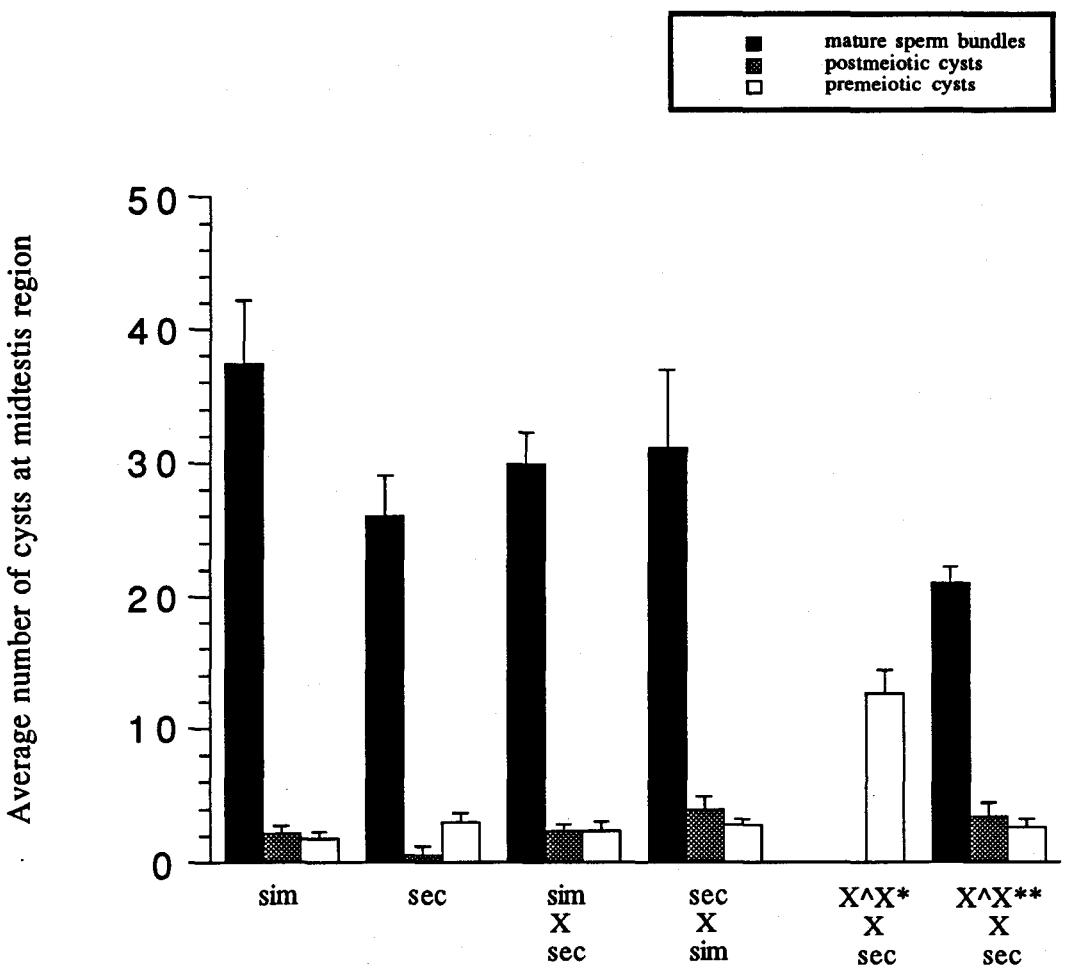
sec refers to *D. sechellia* (3590)

Figure 3.6 Summary of midtestes cyst profile in *sim/sec* hybrids

The type and respective amounts of cysts (including sperm bundles) found at the midtestes level are displayed for the four sets of F_1 interspecific hybrids originating from the cross between *D. simulans* and *D. sechellia* in addition to the two parental species. Five testes, each 66-72 hours old posteclosion, were independently sampled from each set of hybrids. Error bars represent the standard error of the mean for each set of hybrids. The first two sets correspond to the parental lines. The next two sets correspond to the reciprocal crosses while the last two are products of the attached-X cross. The first species listed represents the female of the parental cross.

sim = *D. simulans* (Colombia)
sec = *D. sechellia* (3590)
(X^X)* = *D. simulans (2119)*
(X^X)** = *D. simulans (2119/914)*

The two attached-X lines, with different genetic backgrounds, produce quite different sterility phenotypes when crossed to *D. sechellia.* The origin of the attached-X line, 2119/914, is shown in Figure 2.2 (Materials & Methods).


mature sperm bundles
postmeiotic cysts
premeiotic cysts
Average number of cysts at midtestis region
50
40
30
20
10
0
sim
sec
sim X sec
sec X sim
X^X* X sec
X^X** X sec

3.2.2 Hybrids between *D. simulans* and *D. mauritiana*

In the hybrid genotypes produced from the hybridization of the species, *D. simulans* and *D. mauritiana*, two classes of sterility, premeiotic and postmeiotic, were represented. Hybrids produced from the cross, *D. mauritiana* (female) X *D. simulans* (male), seemed to be identical in sterility to genetically analogous hybrids produced by an attached-X cross (Table 3.4; Figure 3.6; Figure 3.7). These two hybrid genotypes were completely devoid of sperm bundles at all stages of postmeiotic development. The midtestes region was instead full of premeiotic cysts of varying cell stages ranging from two to eight cells each. Sixteen-cell cysts were rarely seen.

In striking contrast to the above aspermic condition, *D. simulans* (female) X *D. mauritiana* (male) hybrids had numerous sperm bundles at the apical, midtestes and basal regions of the testes. Sperm bundles at the midtestes regions were large, diffuse and relatively small in number, compared to the *sim/sec* hybrids.

Table 3.4

Spermatogenic characteristics in interspecific F_1 hybrids of *D. simulans* and *D. mauritiana*

Species Crosses (females X males)	Number of testes examined n	Number of sperm bundles at apex of testis mean (SD)	Presence of elongated sperm bundles at midtestis region	Number of sperm bundles at midtestis region mean (SD)	Number of sperm bundles at midtestis cross-section[a] mean (SD, n)	Number of pre-elongation spermatid cysts at midtestis[a] mean (SD, n)	Number of premeiotic cysts at midtestis[a] mean (SD, n)	Presence of elongated cysts at testis' base	Coiled sperm bundles at testis' base	Sperm bundles at terminal epithelial cell region	Sperm presence in seminal vesicle
D. simulans X D. mauritiana											
sim (Colombia) X mau (LG24)	70	1.03 (1.8)	yes	>8	12.0 (5.9, 5)	2.4 (1.5, 5)	1.4 (0.9, 5)	yes	no	no	no
sim (Colombia) X mau (2252)	74	2.13 (2.3)	yes	>8	-	-	-	yes	no	no	no
sim (South Africa) X mau (LG24)	20	1.30 (1.1)	yes	>8	-	-	-	yes	no	no	no
sim (South Africa) X mau (2252)	36	2.06 (1.4)	yes	>8	-	-	-	yes	no	no	no
D. mauritiana X D. simulans											
mau (LG24) X sim (Colombia)	38	0.0	no	0.0	0.0 (0, 5)	0.0 (0, 5)	8.0 (2.7, 5)	no	no	no	no
mau (LG24) X sim (South Africa)	26	0.0	no	0.0	-	-	-	no	no	no	no
mau (2252) X sim (Colombia)	46	0.0	no	0.0	-	-	-	no	no	no	no
mau (2252) X sim (South Africa)	22	0.0	no	0.0	-	-	-	no	no	no	no
D. simulans(X^X) X D. mauritiana											
sim (X^X-2119) X mau (LG-24)	52	0.0	no	0.0	0.0 (0, 5)	0.0 (0, 5)	6.4 (1.8, 5)	no	no	no	no
sim (X^X-2119) X mau (2252)	68	0.0	no	0.0	-	-	-	no	no	no	no

[a] as in Table 3.2

All other data was collected from observations of whole-mounted testes under Nomarski optics.

Figure 3.7 Testes of *sim/mau* hybrids

Whole-mounted testes observed under Nomarski optics (left) and midtestes cross-sections (right) of F_1 hybrid testes between *D. simulans* and *D. mauritiana.* (A) *D. simulans* (female) X *D. mauritiana* (male) hybrid testes show very conspicuous sperm bundles (arrowheads) from the apical (MT) region through the midtestis region to the testis' base. These sperm bundles are large but distinct. (B) Cross-sections reveal a smaller testis area with a relatively small amount of sperm bundles of various sizes. Seminal vesicle (arrowhead) displays the characteristic lack of sperm found in all hybrids of this study. (C) *D. mauritiana* (female) X *D. simulans* (male) hybrids are completely devoid of sperm bundles as seen in both Nomarski and (D) sectioned photographs. Midtestis' cross-sections reveal that each cyst contains less than 16 cells. (E-F) *D. simulans(X^X)* (female) X *D. mauritiana* (male) hybrids have similar characteristicsas *D. mauritiana* (female) X *D. simulans* (male) hybrids. All rightside panels are to the same scale bar as A, 100 µm. All leftside panels are to the same scale bar as B, 20 µm.

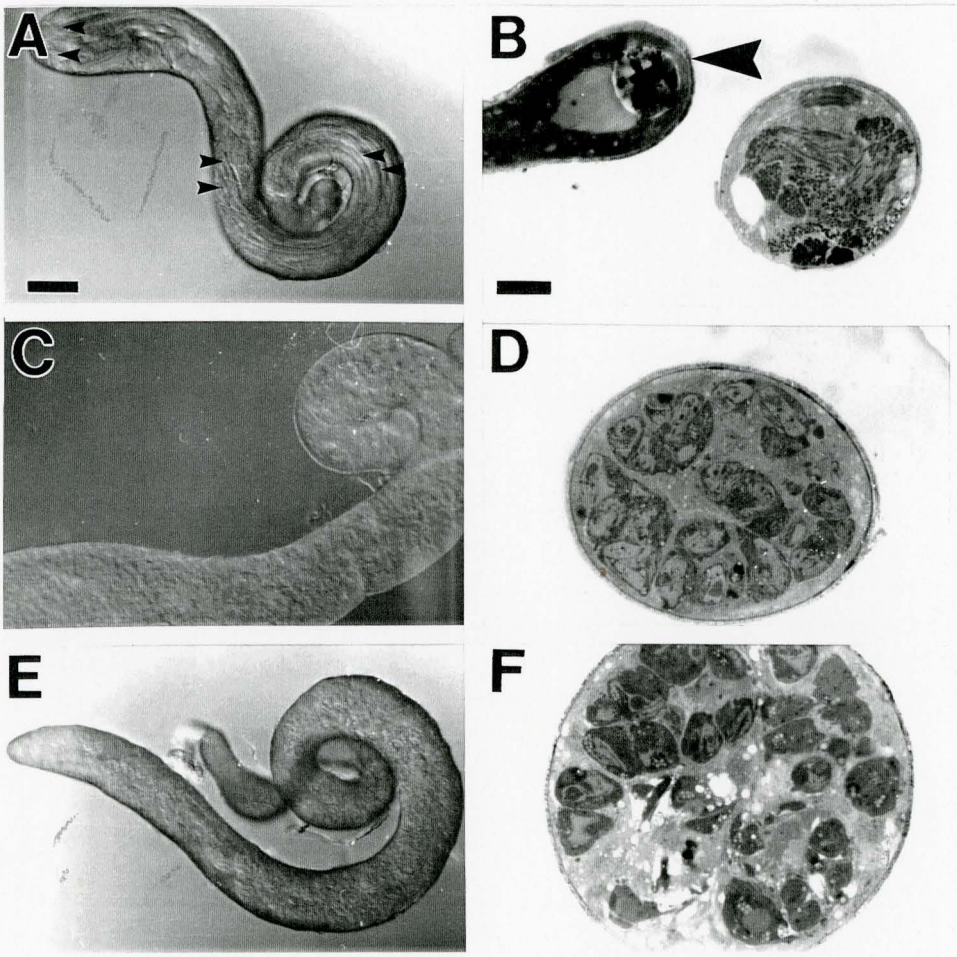
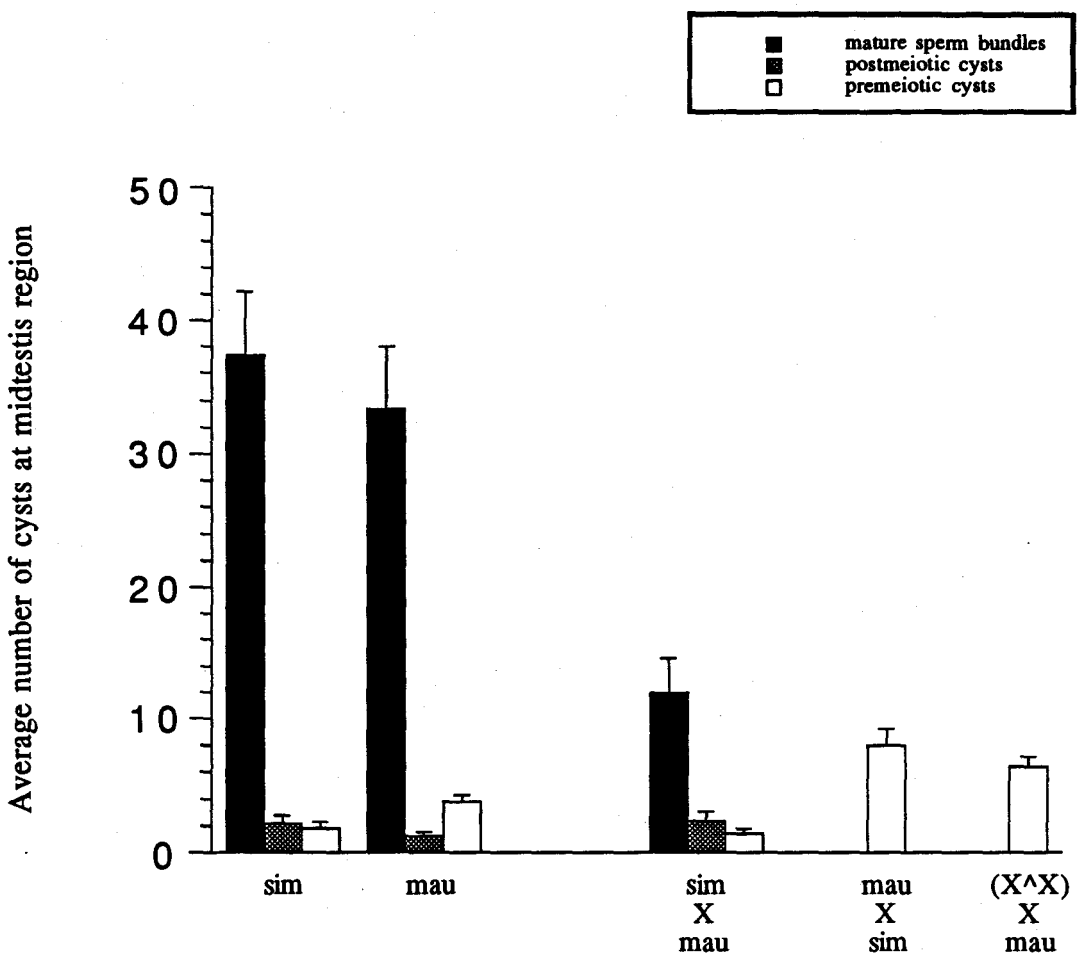

A
B
C
D
E
F

Figure 3.8 Summary of midtestes cyst profile in *sim/mau* hybrids

The type and respective amounts of cysts (including sperm bundles) found at the midtestes level are displayed for the three sets of F_1 interspecific hybrids originating from the cross between *D. simulans* and *D. mauritiana* in addition to the two parental species. Five randomly sampled testes were evaluated for each set of hybrids. Each testis was 66-72 hours old posteclosion. Errors bars represent the standard error of the mean. The first two sets of hybrids correspond to the reciprocal crosses while the last one is the product of the attached-X cross. The first species listed in each cross represents the female parent.

sim = *D. simulans* (Colombia)
mau = *D. mauritiana* (LG24)
sim(X^X) = *D. simulans (2119)*


mature sperm bundles
postmeiotic cysts
premeiotic cysts
Average number of cysts at midtestis region
50
40
30
20
10
0
sim
mau
sim X mau
mau X sim
(X^X) X mau

3.2.3 Hybrids between *D. sechellia* and *D. mauritiana*

The two sets of *sec/mau* hybrid genotypes, originating from each of the two reciprocal crosses, had completely different degrees of sterility (Table 3.5; Figure 3.8; Figure 3.9). The hybrids of the cross, *D. sechellia* (female) X *D. mauritiana* (male), progressed through the postmeiotic stages of spermatogenesis. However, hybrids of this particular cross manifested a very low sperm bundle count at the midtestes region in both 16 hour old and 66-72 hour old adult males.

The reciprocal cross, *D. mauritiana* (female) X *D. sechellia* (male) produced aspermic males with severe sterility defects that were solely premeiotic in nature.

Table 3.5

Spermatogenic characteristics in interspecific F_1 hybrids of *D. sechellia* and *D. mauritiana*

Species Crosses (females X males)	Number of testes examined n	Number of sperm bundles at apex of testis mean (SD)	Presence of elongated sperm bundles at midtestis region	Number of sperm bundles at midtestis region mean (SD)	Number of sperm bundles at midtestis cross-section[a] mean (SD, n)	Number of pre-elongation spermatid cysts at midtestis[a] mean (SD, n)	Number of premeiotic cysts at midtestis[a] mean (SD, n)	Presence of elongated cysts at testis' base	Coiled sperm bundles at testis' base	Sperm bundles at terminal epithelial cell region	Sperm presence in seminal vesicle
D. sechellia X D. mauritiana											
sec (3590) X mau (LG24)	44	0.0	yes	3.86 (2.3)	6.4 (3.2, 5)	3.4 (1.3, 5)	3.6 (1.1, 5)	yes	no	no	no
sec (3590) X mau (2252)	52	0.0	yes	2.76 (2.1)	-	-	-	yes	no	no	no
sec (3588) X mau (LG24)	22	0.0	yes	2.03 (2.7)	-	-	-	yes	no	no	no
sec (3588) X mau (2252)	18	0.0	yes	1.33 (0.8)	-	-	-	yes	no	no	no
D. mauritiana X D. sechellia											
mau (LG24) X sec (3590)	28	0.0	no	0.0	0.0 (0, 5)	0.0 (0, 5)	7.6 (1.8, 5)	no	no	no	no
mau (LG24) X sec (3588)	32	0.0	no	0.0	-	-	-	no	no	no	no
mau (2252) X sec (3590)	22	0.0	no	0.0	-	-	-	no	no	no	no
mau (2252) X sec (3588)	16	0.0	no	0.0	-	-	-	no	no	no	no

[a] as in Table 3.2

All other data was collected from observations of whole-mounted testes under Nomarski optics.

Figure 3.9 Testes of *sec/mau* hybrids

Whole-mounted testes observed under Nomarski optics (left) and midtestes cross-sections (right) of F_1 hybrid testes between *D. sechellia* and *D. mauritiana*. (A) *D. sechellia* (female) X *D. mauritiana* (male) hybrid testes display large, diffuse yet distinct sperm bundles (arrowheads) from the MT to basal regions. (B) These sperm bundles are few in number yet relatively large in size. (C) *D. mauritiana* (female) X *D. sechellia* (male) hybrids are completely devoid of sperm bundles as seen in both Nomarski and (D) sectioned photographs. Midtestis' cross-sections reveal that each cyst contains less than 16 cells each. All rightside panels are to the same scale bar as A, 100 µm. All leftside panels are to the same scale bar as B, 20 µm.

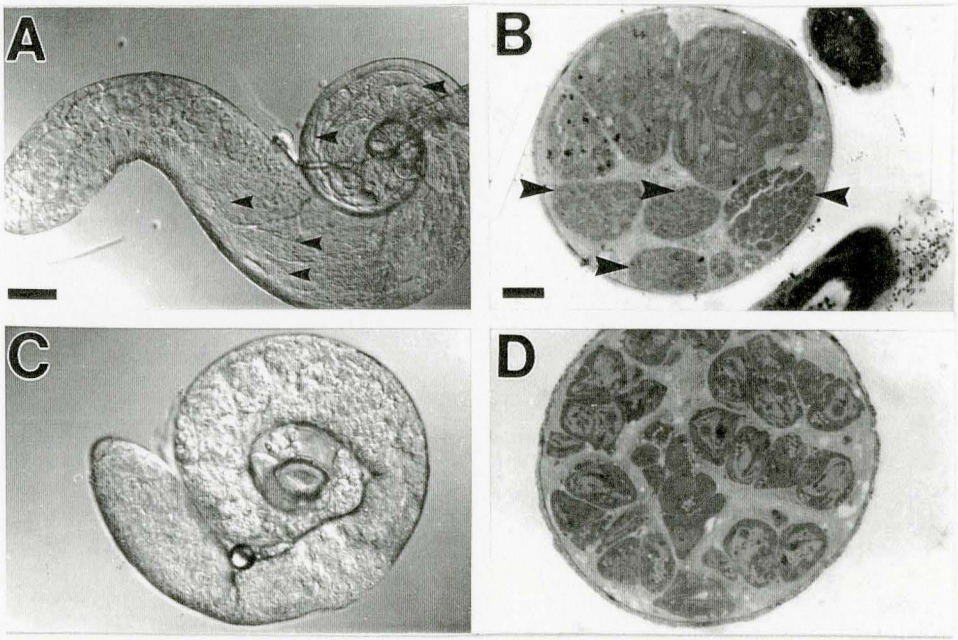
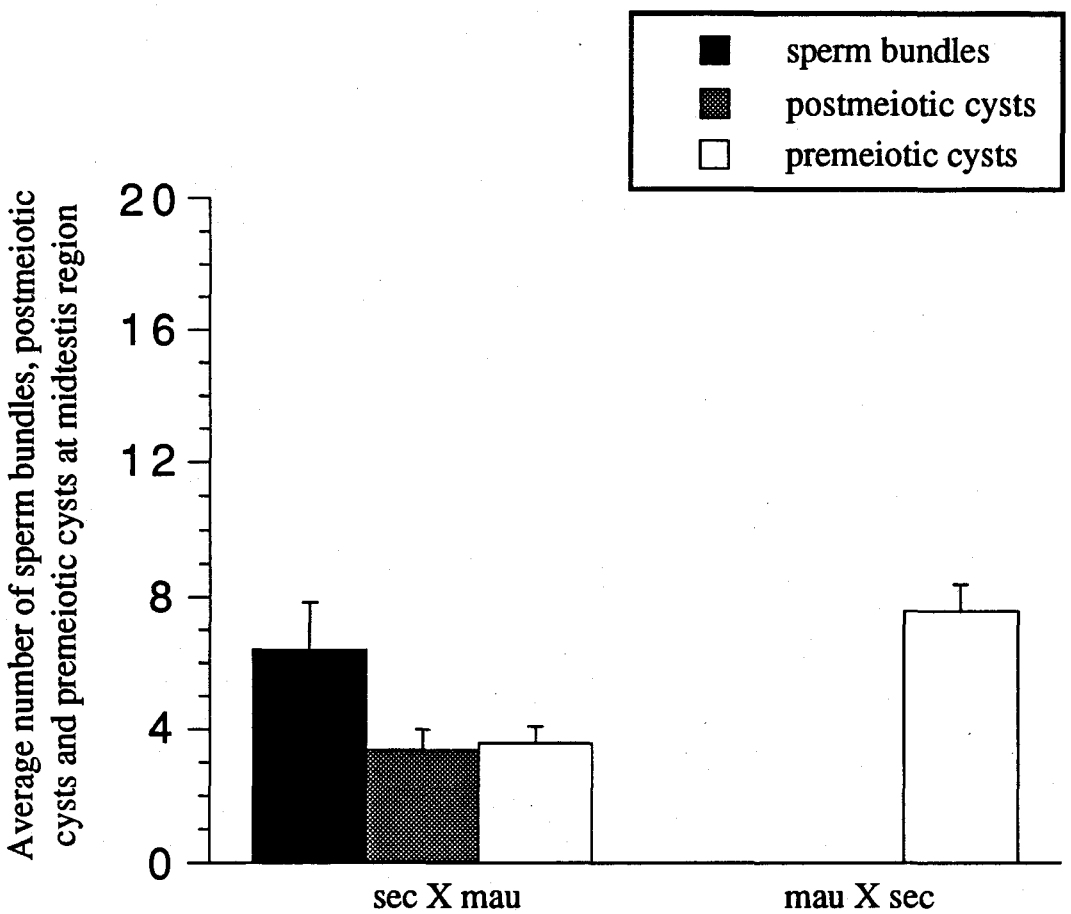

A
B
C
D

Figure 3.10 Summary of midtestes cyst profile in *sec/mau* hybrids

The type and respective amounts of cysts (including sperm bundles) found at the midtestes level are displayed for the two sets of F_1 interspecific hybrids originating from the cross between *D. sechellia* and *D. mauritiana* in addition to the two parental species. Five testes, each 66-72 hours old posteclosion, were independently sampled. Error bars represent the standard error of the mean. These two sets of hybrids correspond to each of the reciprocal crosses. The first species listed in each cross represents the female parent.

sec = *D. sechellia* (3590)
mau = *D. mauritiana* (LG24)


sperm bundles
postmeiotic cysts
premeiotic cysts
Average number of sperm bundles, postmeiotic cysts and premeiotic cysts at midtestis region
20
16
12
8
4
0
sec X mau
mau X sec

3.3 Spermiogenic defects in hybrids

Four out of the six F_1 hybrids, between species of the *simulans* clade, exhibited arrests in spermatogenesis that were postmeiotic in nature. In all hybrids, a large amount of morphological, size, and developmental variation was observed *between* sperm bundle tails of a particular testis cross-section. As well, in contrast to the synchronous development and congruous spermatid morphology found in wildtype testes, hybrids exhibited large variations between spermatids *within* a single sperm bundle, . However, amidst this 'noise', certain consistencies in spermatogenic blockage were observed with each hybrid genotype. Using electron microscopy, the furthest normally developed spermatids of a particular hybrid genotype were observed to be arrested, developmentally, at a common stage of spermiogenesis. The markers of developmental stage, utilized in this assessment, were the behaviours of the axoneme and mitochondrial derivatives as well as microtubule development. Consistencies in developmental abnormalities, through the observations of ultrastructural defects in the most developed spermatids, were also observed in each of the hybrid genotypes.

***D. simulans* (female) X *D. sechellia* (male):**

The presence of morphological spermatid variation within a particular sperm bundle was obvious. Much of this variation may be attributed to the presence of both separated and non-separated spermatids and contrast the monomorphic nature found in normally developing sperm bundles. In these hybrids, the absence of complete cell division between particular spermatids was associated with the lack of clearly discernable cell membranes that are usually formed between neighbouring axonemal complexes. Within such spermatids, large and

varying amounts of ribosome-rich cytoplasm, not typical of normally developing spermatids, were found surrounding axonemal complexes. Thus, amongst neighbouring spermatids, spermatogenic progression is not synchronous with respects to cell division and cytoplasmic removal. Such non-synchronous behaviour contrasts the extreme harmonization found between normally developing spermatids (Fuller, 1993).

Spermiogenesis, in the furthest developed spermatids of this hybrid genotype, appears to be arrested at a pre-individualization stage whereby the paracrystalline material is the same size as the minor mitochondrial derivative. The presence of cytoplasmic material around the axoneme and mitochondrial derivatives as well as the presence of large angles between the axonemal profile axis and the paracrystalline-axonemal axis, indicate the lack of individualization amongst these spermatids (Figure 3.11B).

Axonemal development, although possessing the normal '9+2' microtubule architecture typical of eukaryotic flagellum (Gibbons *et al.*, 1981), revealed developmental abnormalities such as the premature filling (relative to the pre-individualization stage defined by mitochondrial derivative development) of the central singlet microtubule pair with densely staining fibre. As well, densely stained satellite presence and the non-uniform association of the axoneme to its sheath (Figure 3.11C) were also markers of abnormal spermatid growth.

Faintly stained nuclei clusters were observed towards the basal end of orcein-treated testes. Such clusters contained sperm heads of variable length and position. Under a testis squash, stained regions were slender and non-concave (Figure 3.15E). This contrasts the darkly stained nuclei clusters of consistent length and fenestrated shape in wildtype flies (Figure 3.15A-D).

***D. sechellia* (female) X *D. simulans* (male)**

Axonemal complexes within a particular sperm bundle, were observed to exist abnormally in both syncytial and fully separated states (i.e. no evidence of ring canals or other intercellular bridges). However, the developmental timing of spermatogenic arrest in axonemes and their associated mitochondrial derivatives seemed to be fairly consistent during certain spermatogenic stages in neighbouring spermatids of a sperm bundle, no matter their present state of separation (Figure 3.12A). This lack of correlation between separation state and axonemal-mitochondrial derivative development seemed apparent in all hybrids.

The furthest developed spermatids were those that were found during the pre-individualization stages whereby the major and minor mitochondrial derivatives are approximately the same size (Figure 3.12B) and the paracrystalline material begins to offset the minor mitochondrial derivative (Figure 3.12C). The most advanced stage of spermiogenic arrest in this hybrid genotype appeared to take place slightly earlier than that of its reciprocal hybrid (see above). In addition, microtubule development appears less advanced than the reciprocal hybrid of this genotype as the spermatids in these stages possessed evenly spaced microtubules around the mitochondrial derivatives. Such a pattern of microtubule distribution is normally found in elongating spermatids just prior to individualization. In addition, the process of cellular individualization, involving the traversal of cystic bulges, didn't seem to have taken place because of the presence of excess cytoplasm in all spermatids of this hybrid.

Of the four postmeiotic sterile hybrids, this was the only hybrid genotype that did not express stained nuclei heads when treated with orcein. Even under squashed testes conditions (whereby the testes is ruptured and allowed to expel its contents), there seemed to be a

complete lack of stained spermatid nuclei. This absence, however, was not verified through electron microscopic analysis of the head region of the spermatid.

***D. simulans* (female) X *D. mauritiana* (male)**

In addition to the larger size of the sperm bundles, relative to the *sim/sec* hybrids (compare Figure 3.13A to Figures 3.11A and 3.12A), there was a relatively greater area of cytoplasmic debris. The furthest developmental arrests approximately occurred at the developmental stage whereby the paracrystalline material starts to recess around the axoneme and begins to displace the minor mitochondrial derivative (Figure 3.13C). The pre-individualized arrest stage of this hybrid genotype seemed less advanced than the blockage of the previous hybrids (*sim/sec* hybrids) since a relatively larger minor mitochondrial derivative was always present. However, the development of accessory microtubules seemed quite advanced since such structures were absent from these spermatids, indicating post-individualization events. Abnormalities that were consistent with this hybrid genotype included the association of an axoneme to more than one paracrystalline material as well as improper axonemal association to its sheath.

Loose aggregates of orcein-stained nuclei clusters were observed in squashed testes preparations. Such nuclei, however, were rare and faintly coloured. The lack of fenestration, again an indication of abnormal nuclear development, was noted as well.

***D. sechellia* (female) X *D. mauritiana* (male)**

The enormity in sperm bundle size, relative to all the other hybrids with postmeiotic defects, was quite evident (Figure 3.14B). Increases in size seemed to be caused by an associated increase in cytoplasm. Much larger deviations from the normal spermatid complement of sixty-four were also apparent in this hybrid's sperm bundles. Such a decrease in normal spermatid numbers implies the occurrence of spermatid degeneration, and may account for the observed excess in cytoplasm.

Non-syncytial spermatids were common but persisted to be the least developed of the four postmeiotically sterile hybrids. The furthest normally developed spermatids, of this hybrid genotype, contained very immature axonemal complexes, relative to the other hybrids used in this study. Conspicuous minor and major mitochondrial derivatives of similar size as well as relatively small paracrystalline material revealed that spermiogenic arrests took place during an early elongation stage of spermiogenesis. Uneven arrangements of accessory microtubule around the mitochondrial derivatives as well as non-compartmentalization of the axoneme also argued for an early spermiogenic arrest well before individualization. Axonemal abnormalities were abundant and included such anomalies as irregular accessory microtubule deposition, peripheral microtubule accumulation, abnormal sheath associations and multiple paracrystalline attachments to single axonemes (Figure 3.14B,C).

Spermatid head nuclei were observed in orcein treated testes. However, akin to other hybrids, such observations were relatively rare (compared to wildtypes). Hybrids contained a loose assortment of faintly stained and slender rods with no evidence of concavity (Figure 3.15F).

Figure 3.11 Spermiogenic defects in *D. simulans*♀ X *D. sechellia*♂ hybrids

Electron micrographs of testis cross-sections of F_1 hybrids stemming from crosses between *D. simulans* (females) X *D. sechellia* (males). (A) A large variation in morphology and size is apparent amongst sperm bundles. Incomplete individualization can be observed in all spermatids within certain sperm bundles. In many sperm bundles, fully separated spermatids have been displaced outside of their respective sperm bundles (curved arrows). (B) The arrangement of spermatids and their corresponding axonemal complexes within each sperm bundle of this hybrid appears quite irregular. Each axoneme is associated with a darkly stained region called the paracrystalline material which is located within the major mitochondrial derivative. Layers of misplaced endoplasmic reticulum (small arrows) can be observed occupying the sperm bundle. (C) The axoneme (A) is attached to the major mitochondrial derivative (MM) and its associated paracrystalline material (P) in addition to the minor mitochondrial derivative (mM). The conspicuous presence of a minor mitochondrial derivative indicates that the process of individualization has not been completed. Other cross-sections of this hybrid (not shown) reveal the presence of a small but still noticeable mitochondrial derivative. In this micrograph, the angle between the axonemal profile axis (aX) and the paracrystalline-axonemal axis (paX) is approximately 90° indicating a pre-individualized spermatid. Abnormal development is reflected by the absence of accessory microtubules around the perimeter of the mitochondrial derivatives as well as the presence of densely stained satellites on the large arc side of the axoneme (small arrows). However, this may also be a reflection of the progression of certain morphogenetic processes past individualization. In addition, the correspondence of the axoneme to its sheath seems very erratic. A developing appendicular stripe (AS), that usually developsafter individualization, emerges between the axoneme, major mitochondrial derivative and spermatid plasma membrane. Scale bar (A-C); 5 μm, 1 μm, 0.1 μm.

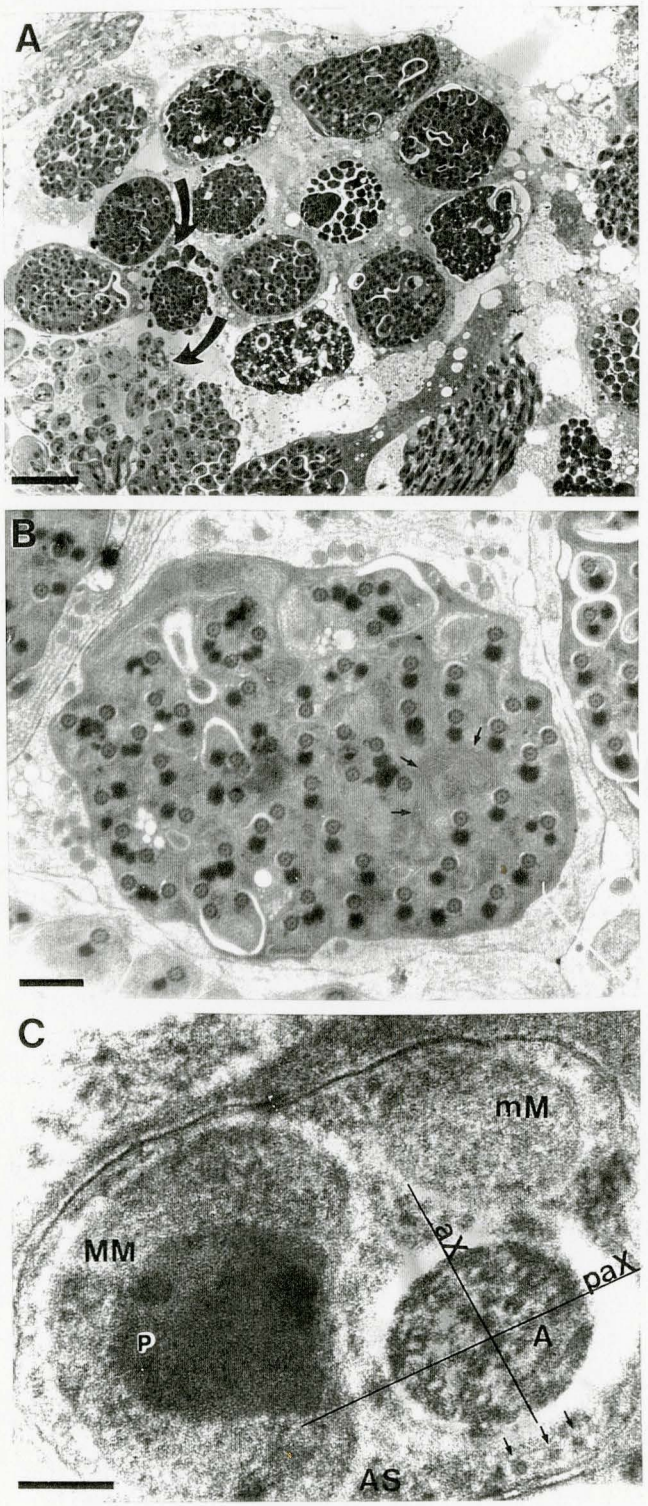

A
B
C
mM
MM
P
aX
paX
A
AS

Figure 3.12 Spermiogenic defects in *D. sechellia*♀ X *D. simulans*♂ hybrids

Electron micrographs of testis cross-sections of F_1 hybrids stemming from crosses between *D. sechellia* (females) X *D. simulans* (males). (A) Variation in sperm bundle size is again apparent in these hybrids in addition to the presence of numerous spermatids that have completed cell division. (B) Large amounts of excess cytoplasm as well as variable spermatid morphologies are observed in this sperm bundle (C) An individually compartmentalized spermatid with a single axonemal complex is found beside two syncytial spermatids that have not undergone separation. A plasma membrane is not observed between the two axonemal complexes of the latter. A number of layers of misplaced endoplasmic reticulum are present (curved arrows). The roughly even-spaced presence of accessory microtubules (small arrows) around the perimeters of the mitochondrial derivatives are indicative of an elongation stage just prior to individualization. Such accessory microtubules originate normally from the peripheral edges of the axoneme (for examples, see arrowheads) of the B-subfibrils of the outer edged microtubule doublets. These axonemal components, as well as numerous electron dense associations between the axoneme and its sheath, are observed in the cross-section indicating a pre-individualization stage of spermatid development. The presence of relatively large minor mitochondrial derivatives (mM), in the mature spermatids of this hybrid, is also representative of the elongation stages. This testis cross-section, however, does not represent the farthest progressed spermatid in this hybrid (data not shown) as minor mitochondrial derivative diminution does takes place in association with a corresponding increase in paracrystalline material. Note the large amount of cytoplasmic material present around each axonemal complex. Scale bar (A-C); 5 μm, 1 μm, 0.1 μm.

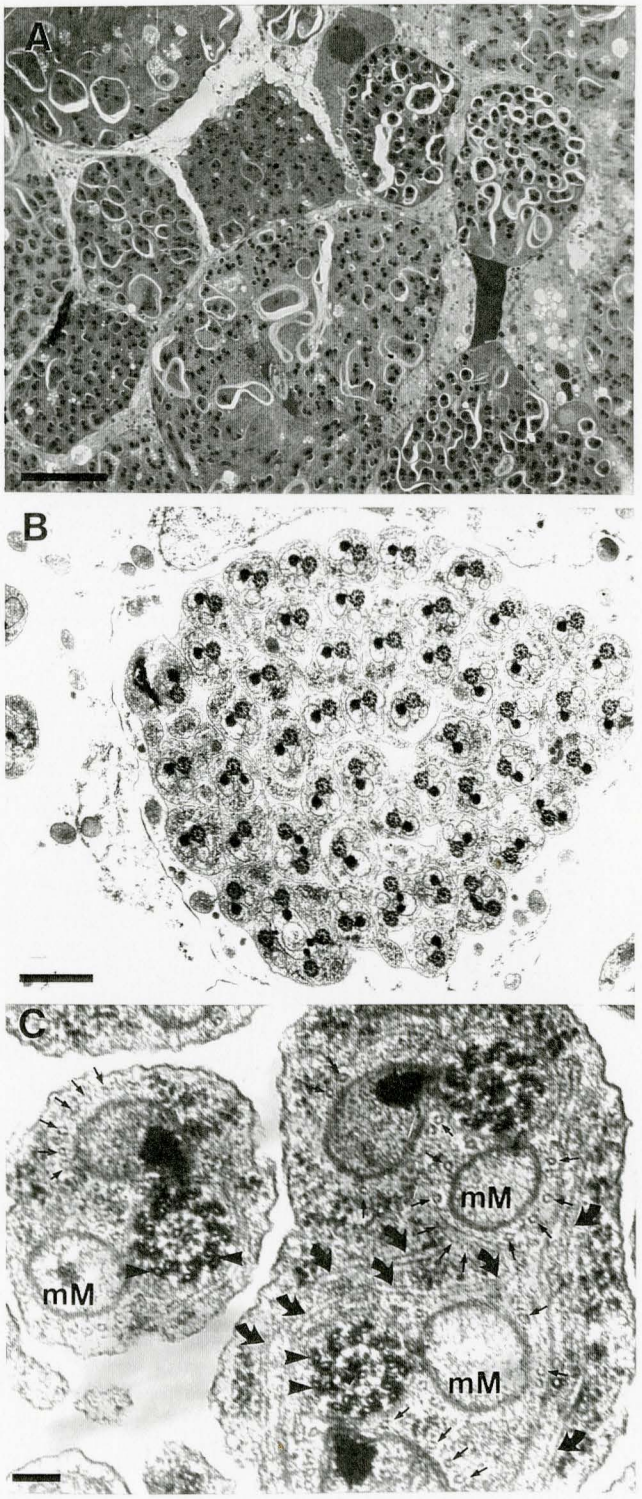

A
B
C
mM
mM
mM

Figure 3.13 Spermiogenic defects in *D. simulans*♀ X *D. mauritiana*♂ hybrids

Electron micrographs of testis cross-sections of F_1 hybrids stemming from crosses between *D. simulans* (females) X *D. mauritiana* (males). (A) Sperm bundles are larger in this particular hybrid compared to the sim/sec hybrids (this micrograph contained the smallest sperm bundles in the cross-section). There seems to be a greater amount of cellular debris between each sperm bundle compared to the sim/sec hybrids. An enormously enlarged mitochondrial whorl is present (large arrow). (B) In this sperm bundle, spermatids that have separated from one another are observed amongst spermatids that have not undergone cell separation although both types seem to procure similar axonemal-mitochondrial derivative relationships. (C) The major mitochondrial derivative and its associated paracrystalline material have developed a recess surrounding the axoneme (between large arrowheads) but have yet to displace the minor mitochondrial derivative (mM). However, the post-individual characteristic of accessory microtubule absence is evident. In addition, the axoneme is partially associated with its surrounding membrane, the axonemal sheath (small arrows), indicating improper axonemal development. Scale bar (A-C); 5 μm, 1 μm, 0.1 μm.

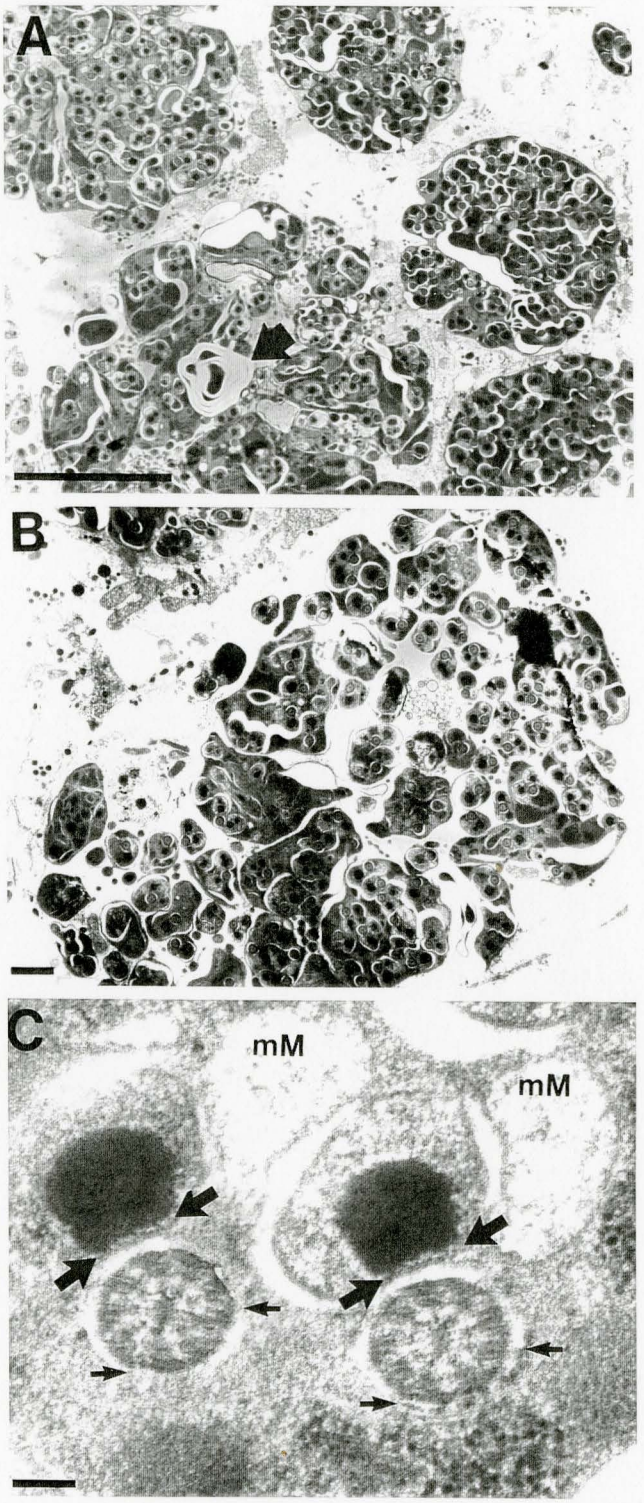

A
B
C
mM
mM

Figure 3.14 Spermiogenic defects in *D. sechellia*♀ X *D. mauritiana*♂ hybrids

Electron micrographs of testis cross-sections of F_1 hybrids stemming from crosses between *D. sechellia* (females) X *D. mauritiana* (males). (A) A transverse cross-section of a sperm bundle in an elongation stage reveals that the progression of spermatid development is not synchronous within a sperm bundle. The absence of neighbouring sperm bundles in this cross-section reflects the relatively low number of sperm bundles found in midtestis cross-sections of this hybrid. (B) Note the large size of the sperm bundle, typical of this hybrid, compared to that of other hybrids. A large amount of cellular debris is found between spermatids. The number of axonemal complexes is significantly less than the normal complement of sixty-four, found in normally developing sperm bundles. Hence possibly, the degeneration of spermatids may have lead to this excess in debris. Syncytially separated spermatids are observed amongst spermatids that have yet to separate. (C) The asymmetrical presence of large numbers of accessory microtubules around the perimeter of both mitochondrial derivatives is indicative of an early stage in spermatid elongation (small arrows). Accessory tubules are observed to originate on the periphery of the axoneme and can be seen normally separating from the axonemal sheath (large arrows) of this spermatid. However, accumulation of accessory microtubules (curved arrows) is representative of abnormal spermatid development. Non-compartmentalization of the axoneme in its sheath also indicates incomplete development. Note the large size of the minor mitochondrial derivative (mM), found in the most mature spermatids of this hybrid. Scale bar (A-C); 5 μm, 1 μm, 0.1 μm.

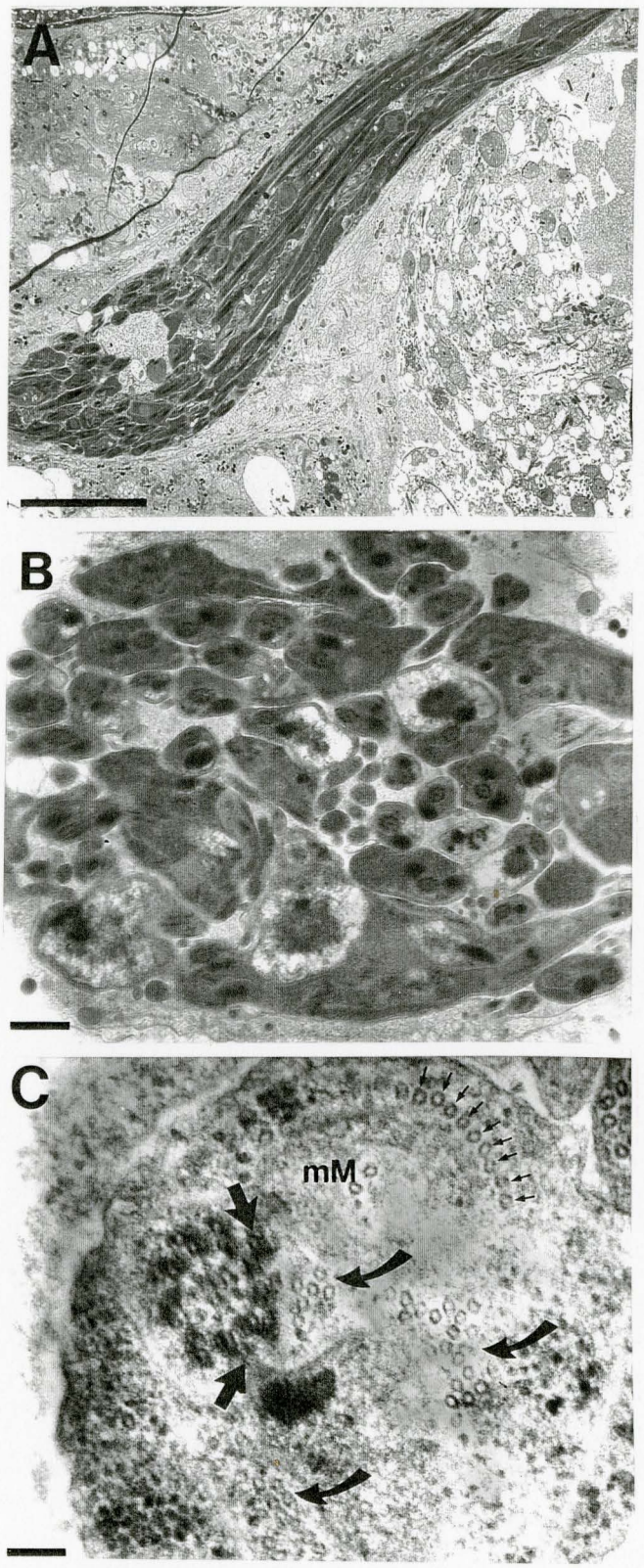

A
B
C
mM

Figure 3.15 Spermatid nuclei presence in postmeiotically arrested hybrids

Utilizing an orcein staining protocol, the presence or absence of spermatid head nuclei is assessed in sperm bundles. (A) In *D. simulans,* as well as other species of the *simulans* clade, the conspicuous presence of brightly stained nuclei clusters (small arrows) is ubiquitous. Such densely stained nuclei are generally observed in the basal half of the testis lumen just before coiling takes place. By changing the depth plane in the field of view, other clusters could be observed. Testis was observed under brightfield. Scale bar, 100 μm. (B) High magnification view of densely stained nuclei in *D. simulans* in testis lumen. Scale bar, 20 μm. (C-D) Ruptured testis of *D. simulans* display a number of nuclei clusters. Note the difference in nuclei morphologies between the different clusters denoting differing stages of nuclei development. The more developed nuclei have acquired a slight concavity at its fenestrated side (small arrowheads). (E-F) Interspecific hybrids stemming from crosses *D. simulans*(female) X *D. sechellia* (male) and *D. sechellia* (female) X *D. mauritiana* (male) , respectively. Nuclei cluster (large arrows) are rare and are not clearly defined. In unruptured testes profiles, nuclei are difficult to observe since they are very lightly stained. Spermatids within a cyst may contain stained regions that are found in areas outside of the nuclei cluster (arrowheads). In addition, stained regions of each spermatid within each hybrid cyst are of variable lengths. The majority of stained nuclei do not exhibit any concavity. Stained nuclei clusters were also observed in *D. simulans* (female) X *D. mauritiana* (male) hybrids. None, however, were observed in *D. sechellia* (female) X *D. simulans* (male) hybrids. Panels C-F to the same scale bar as B, 20 μm.

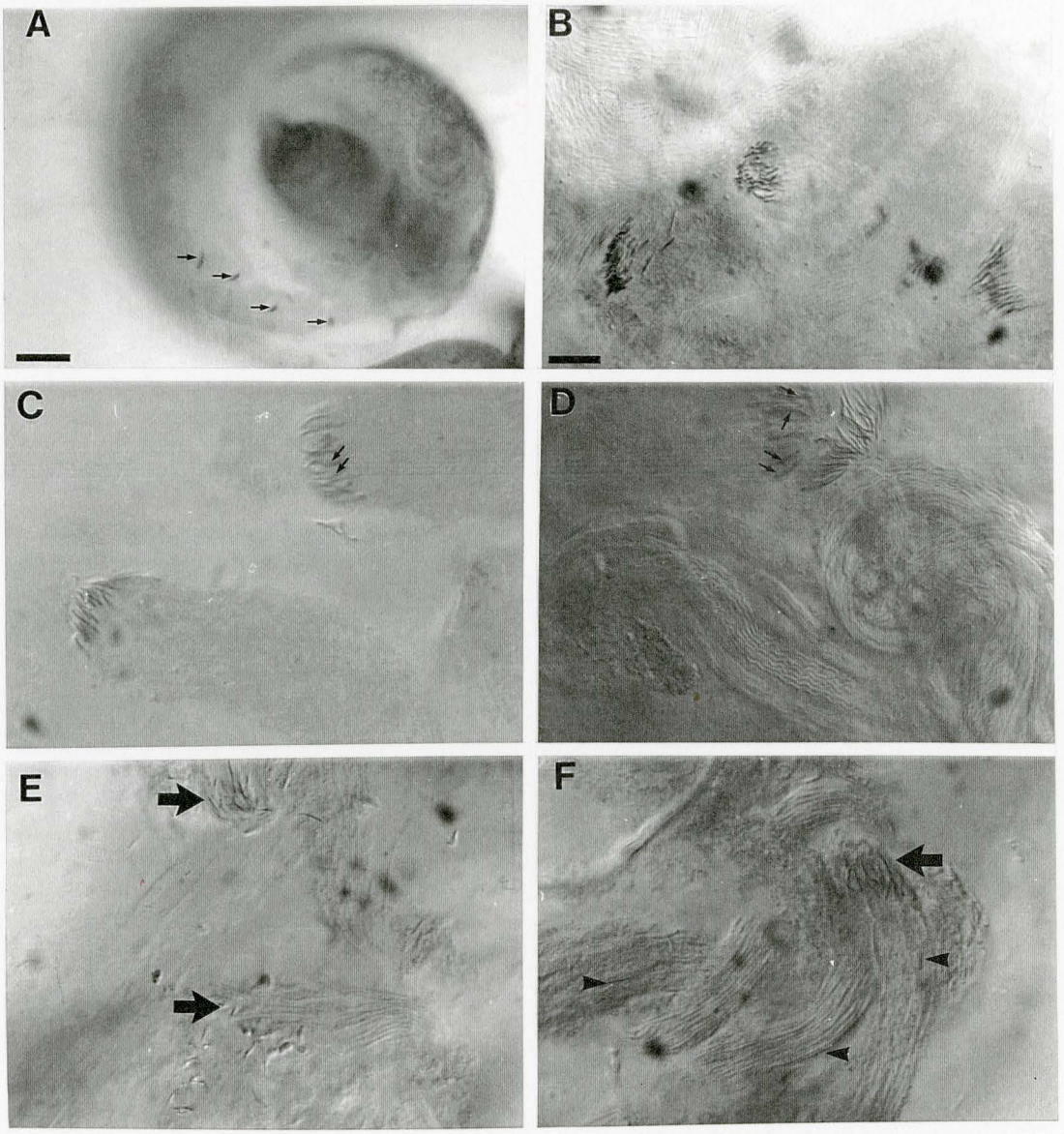

A
B
C
D
E
F

Table 3.6

Summary of hybrid male sterility defects in the *simulans* clade

Parental type	***D. simulans*** ♀	***D. sechellia*** ♀	***D. mauritiana*** ♀
D. simulans ♂	fertile	Postmeiotic sterility -large number of sperm bundles -sperm bundle degeneration (after 3 days) -preindividualized axonemal complex -immature microtubule development	Premeiotic sterility -two to eight cell cysts
D. sechellia ♂	Postmeiotic sterility -large number of sperm bundles -preindividualization axonemal complex -most mature spermatid development -mature microtubule development	fertile	Premeiotic sterility -two to eight cell cysts
D. mauritiana ♂	Postmeiotic sterility -small number of sperm bundles -preindividualized axonemal complex -large minor mitochondrial derivatives -abnormal paracrystalline development	Postmeiotic sterility -small number of sperm bundles -early elongation spermatids -immature axial development -large amount of spermatid degeneration	fertile

Discussion

4.1 Spermatogenesis in the *melanogaster* complex

The developmental process of spermatogenesis appears to be well conserved among members of the *melanogaster* complex. In all four of the sibling species employed in this study, sperm bundle elongation seems to commence at the midtestes region and coiling takes place at the basal end of the testis. As well, there are four gonial divisions that result in a sperm bundle of sixty-four spermatids. This latter characteristic contrasts with many other Drosophilid species. For example, *D. hydei* has only three gonial mitoses (Hennig, 1985) while *D. pseudoobscura* and *D. miranda* each produce five gonial divisions (Dobzhansky, 1934; Philip, 1944).

Differences, however, were observed between *D. sechellia* and its siblings. In *D. sechellia*, the amount of midtestes sperm bundles (three days old) was lower than the other species of the complex. This difference was amplified when sperm bundles in *D. sechellia* and *D. simulans* were compared in flies less than two hours old. Another difference between *D. sechellia* and the other species was the near absence of apical sperm bundles found in *D. sechellia*. Longer testis length in *D. sechellia* relative to the other siblings of its clade (A. Civetta, personal communication) may account for the lack of sperm bundles at the testis apex. However, Joly (1987) observed a significantly larger cyst length in *D. sechellia*

compared to that of *D. simulans* and *D. mauritiana*. Thus, the existence of other factors, such as sperm bundle coiling frequency and elongation timing, may cause a deficiency in apical sperm bundle number.

Lower sperm bundle number in *D. sechellia*, relative to the other species of the complex, parallels similar differences in females. The findings of Lachaise *et al.* (1986) showed that the number of ovarioles was much smaller in *D. sechellia* than other species of the complex. In a similar study, Coyne *et al.* (1991) revealed almost identical results in ovariole difference between *D. simulans* and *D. sechellia* to that of Lachaise *et al.* and commented that this low ovariole number in *D. sechellia* was either a true adaptation or simply a byproduct of some other evolutionary change. An analogous argument can be presented with respect to sperm bundle number. Does the difference in sperm bundle number represent a certain adaptation or is it the result of some other factors such as pleiotropy or random genetic drift? The answer to such a question may explain why reproductive characters, in general, seem to differ the greatest between closely related species.

There appeared to be a large amount of intraspecific variation in sperm bundle number, both within strain and between strains of the same species (Table 3.1, Figure 3.2). However, unlike sperm bundles in males, females produce a more-or-less constant amount of ovarioles per ovary (Lachaise *et al.*, 1986; Coyne *et al.*, 1991). Such a consistency may indicate that ovariole number maintains a remarkably higher degree of developmental homeostasis, in comparison with sperm bundle number.

4.2 Spermatogenic defects in hybrid male sterility

Although the overall process of spermatogenesis is indistinguishable between the employed species, all of its male F_1 hybrids are sterile (Lachaise *et al.*, 1986). However, the precise characterization of a particular F_1 hybrid male sterility phenotype is difficult to perform, especially at the ultrastructural level, because of the large number of interactions present, the large number of spermatogenic landmarks present and the presence of within testes variation of cyst abnormalities. Despite these difficulties, large differences in the sterility phenotype between most of the hybrid genotypes were observed in this study (Table 3.6). Such differences are expected since the interactions that produce these sterility incompatibilities evolve independently from each other. Hence, different sets of interacting loci are assumed to affect each interspecific hybrid genotype. Two distinct classes of defects, premeiotic and postmeiotic, were observed amongst the six F_1 hybrid genotypes (Table 3.6). Two hybrids exhibited defects that were premeiotic in nature. Hybrids of the crosses, *D. mauritiana* (female) X *D. simulans* (male) and *D. mauritiana* (female) X *D. sechellia* (male), presented almost identical sterility phenotypes. In both cases, 16-cell cysts were rarely observed and if they were, they were usually extremely malformed. Cells within cysts were very large and similar in size and displayed large variations in cell and nuclear morphology. However, the presumed similarity may simply be a result of the absence of further investigation.

Although abnormalities seemed to take place well before meiosis starts, there still exists the possibility that meiotic processes may be a source of the defects in these hybrids. Since female hybrids of this clade are known to be fertile (Lachaise *et al.*, 1988), this results

shows that there may be inherent genetic differences between the meiotic processes found in males and females. In fact, many male-specific meiotic mutants have been isolated (reviewed in Fuller, 1993). In a study using EMS treated sperm, Bakken (1970) isolated 98 recessive female sterilizing autosomal mutations and found that 24 proved to be male sterile. However, in every single case, Bakken was able to segregate the factor that caused male sterility from the female-sterilizing factor by recombination. This study shows that the two meioses are independent processes that do not show significant overlap.

Four out of the six hybrids exhibited defects that were spermiogenic, or postmeiotic in nature. It must be noted, however, that although these hybrids have reached the postmeiotic stages of spermatogenesis, the existence of premeiotic lesions is still a possibility. In fact, all of these hybrid genotypes displayed a wide range of abnormalities from sperm bundle dynamics to ultrastructural defects. Drastic reductions in sperm bundle number, as well as increases in size, were seen in both hybrids of *D. mauritiana* that had postmeiotic defects. This may indicate that the program of spermatogenesis, in these hybrids, had also been perturbed at an early stage.

Since all of the male hybrids share the characteristic of sterility, some commonalities were expected to exist. None of the hybrids contained sperm in the seminal vesicles. As well, there was an absence of coiling in all of the hybrid genotypes. The absence of this process at the basal end of the testis corresponded to the immature development of spermatid heads (assayed in orcein stains). Hence, the lack of coiling may be due to the failure of spermatid heads to embed themselves into individual columnar terminal epithelial cells situated at the basal end of the testis (Tokuyasu, 1972b). There also seems to be an absence of complete

individualization in all of the hybrids. All postmeiotically defective hybrids produced spermatids with incomplete membrane formation (due to incomplete cell division) and an excess of cytoplasm. As well, mitochondrial derivative and axonemal development were arrested at pre-individualization stages.

Differences were also seen between postmeiotically defective F_1 hybrid genotypes. The degeneration of sperm bundles was recorded in older *D. sechellia* (female) X *D. simulans* (male) hybrids in contrast to a seemingly gradual increase in sperm bundle number in the reciprocal cross. This increase may indicate the continuance of a dynamic spermatogenic steady state (Fuller, 1993). Significant differences in midtestes sperm bundle content between the *mau/sim* and *mau/sec* postmeiotic hybrids were also observed. And when spermatids that developed normally the furthest were compared from each hybrid genotype, differences were again quite evident (Table 3.6)

It is interesting that the majority of defects, in this group of closely related species, manifest themselves postmeiotically. This parallels the pattern observed in within-species sterility (Lindsley & Lifschytz, 1972). Of 144 X-linked male steriles analysed by Lifschytz & Lindsley (unpublished), 132 mutations produced postmeiotic abnormalities such as disrupted spermatid elongation (10), effected failures in sperm maturation (90) and prevented normal sperm transmission (32) (Lifschytz, 1987).

In addition, the diverse array of defects, that was found in hybrid male sterility, also occur in within-species male sterile mutants and may be the consequence of the many events found in spermatid differentiation occurring through independent pathways (Lifschytz, 1987). For example, in the hybrid, *D. simulans-2119* (female) X *D. sechellia* (male), elongation was

still taking place even though the completion of normal meiosis had not. At the substructural level, early defects are difficult to resolve and numerous mutants share "a common classic male sterile phenotype when examined by light microscopy" (Fuller, 1993). These early defect phenotypes include flagellar bundles that elongated but were disorganized and spermatids that degenerated prior to individualization (Hackenstein, 1991; Castrillon *et al.*, 1993). At the ultrastructural level, such male sterile mutants exhibited flagella with disorganized axonemal components and oddly shaped mitochondrial derivatives (Fuller, 1993). The sterility phenotypes found in postmeiotically defective hybrids, in the present study, may also fall into the definition of common classic male sterile phenotype. However, this description is not important and essentially corresponds to our lack of understanding of the various pathways involved in normal spermatogenesis.

The complexity in hybrid spermiogenic sterility may be further instigated by the apparent lack of transcription in the later stages of spermatogenesis. The autoradiographic studies of Oliveri & Oliveri (1965) and Gould-Somero & Holland (1974) showed that [^{3}H]uridine incorporation does not take place in germline cells after the late primary spermatocyte stage indicating that the bulk of RNA synthesis transpires before meiosis. It has also been shown that the presence of chromosomes are unnecessary during spermiogenesis (Lindsley & Tokuyasu, 1980). Hence, the lack of compensatory mechanisms in the form of transcriptional regulation that are important in other developmental processes, "may render spermiogenesis more sensitive to minor stoichiometric changes in gene products" (Wu & Davis, 1993)

Perez *et al.* (1993) stated that the complexity of F_1 hybrid male sterility suggests that

it may be due to the interaction of many genes. However, the complexity of hybrid male sterility does not necessarily establish the presence of a large number of incompatible interactions caused by a large number of genes (polygenic nature) or strong epistatic interactions ("complex epistasis") but may simply be the byproduct of a few or even a single genetic incompatibility on numerous independent pathways.

4.3 Characteristic sterility attributed to each hybrid genotype

Differences between most of the F_1 hybrid genotypes in this study were observed in both substructural (light microscopy) and ultrastructural (electron microscopy) analyses. (Differences between the two premeiotically arrested hybrids were not scored.) Using light microscopy, each hybrid genotype was found to exhibit a characteristic and consistent sterility phenotype. When sterility effects such as sperm bundle number and spermatogenic landmark presence were assayed at the substructural level, consistencies in each interspecific hybrid were observed even when different strains were employed (Tables 3.2, 3.4, 3.5). Thus, in addition to the constancy of sterility between individuals of a certain strain, any variation found between different strains did not seem to manifest noticeable effects on the sterility of the hybrids. (An exceptional strain was discovered in *D. simulans* (2119) that caused a large difference in the sterility of the progeny. This result can not be further explained at this time and will be part of the discussion below.)

Electron microscopic analyses also revealed that particular hybrid genotypes maintain characteristic ultrastructural defects. However, the complex nature of F_1 hybrid sterility seemed to act as a distracting force as variation within each individual testes was in fact

observed. For example, in the spermiogenic defective hybrids, the numerous sperm bundles present may have been in different stages of spermiogenesis. But by only observing the spermatids which have normally developed the furthest, the same stage of spermatogenic arrest, as well as the same set of abnormalities, were consistently observed in each hybrid genotype (Table 3.6).

These results differ from other studies that have attempted to characterize hybrid male sterility amongst the *simulans* clade. Lachaise *et al.* (1986) described the presence of fully developed (yet aspermic) testes in *sim/sec* and *sim/mau* F_1 hybrids and reported a wide range of testicular phenotypes ranging from atrophied testes to fully developed (yet aspermic) testes in F_1 hybrids of the *sec/mau* species pair. Such characterizations, although observed at a lower level of resolution, argues that hybrid sterilities are of a general and common nature. In contrast, the present study relates concrete spermatogenic anomalies to particular hybrid genotypes. A number of factors including environmental differences such as temperature, humidity and rearing medium as well as strain dependent differences may account for the disparity in observations. In addition, the consistent character of sterility that can be denoted to each F_1 hybrid type in the present study may reveal inherent differences between hybrid male sterility and hybrid testes morphology.

Other studies have also briefly characterized certain hybrids of the *simulans* clade. Observing the two F_1 hybrids stemming from the crosses, *D. simulans* (female) X *D. sechellia* (male) and *D. simulans*-*X^X* (female) X *D. sechellia* (male), Coyne & Kreitman (1986) noted that the former hybrid produced 4.2 % motile sperm while the latter attached-X cross did not produce any sperm. Perez *et al.* (1993) observed that hybrids of *D. simulans* (females) X

D. mauritiana (males) progressed through spermatogenesis until the elongation stage of spermiogenesis while hybrids produced from *D. simulans* (females) X *D. sechellia* (males) contained many abnormal onion cells (pre-elongation cysts) and appeared to have enlarged mitochondrial derivatives. Such studies are inconsistent with the present study and may seem to imply the previous absence of a comprehensive evaluation on the nature of hybrid male sterility in this clade, as well as other clades in *Drosophila*.

4.4 Step-wise progression of hybrid male sterility

The constancy of hybrid male sterility, characterized in this study, differs from current opinions on the plasticity of hybrid sterility. In their recovery of female fertility between *D. melanogaster* and *D. simulans*, Davis *et al.* (1996) noted large line-to-line variations in the average 'fertility' of the hybrids. They attribute these results to a "strain-dependent continuum of hybrid female fertility". In this study, such large 'continuums' were generally not observed in the sterilities of the hybrid male (Tables 3.2, 3.4, 3.5) although variations between parental strains had been recorded. Hence, the variations in spermatogenic markers found between strains seemed to have become buffered, and not accentuated, in hybrid sterility. The only strain that created a noticeably different sterility phenotype was the *D. simulans-2119* attached-X line. It was observed that a locus (or loci) from *D. simulans-2119*, when interacting with the genome of *D. sechellia-3590*, produced a much more severe sterility phenotype than the same locus from *D. simulans*-South Africa. The reversion of the premeiotic defect (found in hybrids of *D. simulans-2119* (female) X *D. sechellia* (male)) to a postmeiotic defect was accomplished by substituting the attached-X line with a South

African background and then crossing this novel line to *D. sechellia* (3590). Such a large change in the hybrid's sterility phenotype can not be considered continuous but rather, discrete, and may be based on the influence of loci with large effect. The question must be asked whether this mode of change is common during the postzygotic reproductive isolation of related species.

Interestingly, *intra*specific hybrids of *D. simulans-2119* seemed to be fertile (Table 3.1) although the extent of their fertilities was not assayed. This may be due to species-compatible modifier loci that have evolved in *D. simulans-2119* to counteract the effect of this novel factor(s). In fact, the number of midtestes sperm bundles of the cross, *D. simulans-2119/914* (female) X *D. sechellia* (male), was shown to be significantly lower than genotypically equivalent hybrids of the cross, *D. sechellia* (female) X *D. simulans* (male) (Table 3.6). It is possible that the presence of non-recombined modifier loci in the line was causing this 'decrease in fertility'.

The existence of epistatic fertility loci in hybrid male sterility has recently been shown (Perez *et al.*, 1993: Cabot *et al.*, 1994). Perez & Wu (1995) observed that the *Odysseus* locus alone did not confer sterility upon *sim/mau* hybrids but was dependent on the presence of other neighbouring loci. They concluded that between closely related species, reproductive isolation is usually caused by several genes of weak effect that interact epistatically to create a large effect. However, analyses involving introgressed segments through repeated backcrossing may prove to be illusive since such inferences are based on a reductionist approach in explaining F_1 hybrid male sterility. The *complete* interactions between *all* heterospecific loci, that result in the sterility of the F_1 hybrid, are not evaluated. Rather, this

conclusion is based on a select group of loci that are anomalously placed together in backcrossed males. However such hypotheses, although differing in the cause of reproductive isolation (ie. strong vs. weak allelic effects), does not reject the possibility that the progression of hybrid male sterility can phenotypically occur in discrete steps.

The magnitude of the difference in sterility, within each of the three sets of reciprocal crosses (whereby the parental types are interchanged), differed drastically . There were large differences of premeiotic vs. postmeiotic defects in the *sim/mau* and *sec/mau* hybrid species pairs while sperm bundle degeneration was the major difference between sterility phenotypes of the *sim/sec* F_1 reciprocal hybrids (in testes older than three days old). Such a large difference, amongst three species pairs with recent divergence times, forces us to again evaluate a discrete, or step-wise nature of hybrid male sterility progression. Under a polygenic model of speciation, such large differences would not be expected. However, the accumulation of incompatibilities, from a primary cladogenic event, may have allowed for this large observational difference under such a model.

4.5 Effect of cytoplasm on hybrid male sterility

As stated above, progeny of a certain attached-X cross were observed to possess a different sterility phenotype, compared to their reciprocal hybrid genotypic equivalents. The presumed cause of this difference was a factor stemming from the cytoplasm of *D. simulans*. In this study, cytoplasmic influences were assessed in two F_1 hybrids of the *simulans* clade using an attached-X line of *D. simulans*. This analysis not only allowed cytoplasmic factors to be investigated, but also allowed for the assessment of the second component of cytoplasm

influence - maternal factors (see **1.8**). The sole availability of the compound-X chromosome in *D. simulans* only permitted for unidirectional evaluations of cytoplasmic influence when males of *D. mauritiana* and *D. sechellia* were crossed to *D. simulans-X^X* females. Thus the 'reciprocal' decoupling of cytoplasm from the X-chromosome of *D. sechellia*, as well as *D. mauritiana*, could not be performed. In addition, using this assay, cytoplasmic influences could not be tested between hybrids of the species pair, *D. sechellia* and *D. mauritiana*.

Zeng and Singh (1993), using a recurrent backcross technique, were able to show that cytoplasmic factors do not play a role in hybrid male sterility in the *simulans* clade. However, this method does not allow for the evaluation of maternal effects which are a vital component of cytoplasmic influence. The use of attached-X lines allows such maternal effects to be disentangled from its natural co-transmission with the X-chromosome. Hence, components of the cytoplasm such as maternal effects which, in contrast to cytoplasmic factors, are important to the classical model of speciation (Dobzhansky, 1970; Goulielmos & Zouros, 1995), can be separated from other factors involved in hybrid male sterility.

The presence of both cytoplasmic and maternal effect factors of a *D. simulans* origin, that may affect the sterilities of *sim/mau* F_1 males, was not observed. The sterility phenotype of the two hybrids, *D. simulans-X^X* (female) X *D. mauritiana* (male) and *D. mauritiana* (female) X *D. simulans* (male), was practically identical in their aspermic nature. This indicates the absence of factors transmitted through the cytoplasm (maternal effect or cytoplasmic factors) of *D. simulans-2119* that can interact with an autosome or X-chromosome of *D. mauritiana* to further increase the hybrid's sterility (at least at a detectable level). Davis *et al.* (1994) used these same two species, as well, to show that the cytoplasm

of *D. simulans* does not effect hybrid male sterility. Using an attached-X cross, they produced F_2 male progeny with a *D. simulans* cytoplasm, sex chromosomes of *D. mauritiana* in origin, and varying degrees of *D. simulans* autosomal material. They found that a small proportion of F_2 males were fertile and showed that all of these had autosomes with *D. mauritiana* markers thus proving the existence of fertile hybrids with a *D. mauritiana* genetic background and an introgressed *D. simulans* cytoplasm.

The effect of *D. simulans* cytoplasm on hybrid male sterility in hybrids of the *sim/sec* species pair was also investigated in this study. A large difference in the sterility phenotype was observed between the two hybrids of the crosses, *D. simulans-X^X* (female) X *D. sechellia* (male) and its genotypic equivalent, *D. sechellia* (female) X *D. simulans*-Colombia (male). The former hybrid manifested arrests in spermatogenesis that are solely premeiotic while the latter hybrid displayed postmeiotic abnormalities. This large difference in sterilities may, at first, seem to indicate a cytoplasmic influence (see hybrid schematic in Figure 3.3). However, a *D. simulans* line-to-line difference between *2119* (attached-X) and *0251.2* (Colombia) may have also accounted for this difference. This latter hypothesis was indeed validated by backcrossing female progeny from a *D. simulans-2119* X *D. simulans-914* cross to *D. simulans-914* males for two generations, and crossing the resulting female to a *D. sechellia* male and observing the presence of *post*meiotic defects in cross-sections of the hybrid testes (see above). Thus the influence of both cytoplasmic and maternal effects on hybrid male sterility was deemed uncommon, at least in the *simulans* clade.

It must be noted that the *complete* isolation of maternal effects is not technically feasible using an attached-X crossing scheme. The reason for this can be seen in Figure 2.2.

Since the compound-X chromosome remains in the lineage, the X-chromosomal component of the maternal effect will *always* be of a *D. simulans* origin, even after an infinite amount of backcrosses.

A simple analysis, employing members of other groups that produce sterile hybrid females (the *simulans* clade produces fertile females), may indirectly evaluate such cytoplasmic effects on hybrid male sterility by observing the effect of such factors on hybrid *female* sterility. Although of a completely different genetic program, such an analysis may reveal the overall importance of these factors in hybrid sterility in general. Such an analysis makes use of the fact that reciprocal hybrid females, unlike their male counterparts, are genotypically the same except for the presence of non-nuclear factors. This analysis may be accomplished by calculating the number of interspecific crosses with a reproductive isolation (R.I.) index value of 0.75 (both reciprocal male F_1 hybrids are sterile, one of the two reciprocal F_1 females are sterile) and comparing this quantity to the number of species crosses with an R.I. value of 1.00 (all four F_1 hybrids are sterile). The frequency of occurrence of the former (R.I. = 0.75) class, relative to the class of hybridizations with complete hybrid sterility R.I. = 1.00), will indicate the degree that the cytoplasm has some sort of effect on female sterility. As stated before, such an inference can be made since the only difference between reciprocal females is the origin of their respective cytoplasms. A large survey of diverse species of *Drosophila* has in fact been reported (Coyne & Orr, 1989b). A brief reanalysis of their data reveals that twenty species pairs had R.I. values of 1.00 while nine had R.I. values of 0.75. This indicates that the cytoplasm may in fact have an important influence on hybrid *female* sterility in many groups of drosophilids.

4.6 Asymmetries in sterility between reciprocal crosses

Differences in the sterility phenotype were strikingly apparent *between* all reciprocal crosses within the three species pairs utilized in the study. These asymmetries ranged from the premeiotic vs. postmeiotic anomalies observed in reciprocal F_1 hybrids of the *sim/mau* and *sec/mau* species pairs to the differential degradation of postmeiotic cysts observed in *sim/sec* F_1 hybrids.

Non-reciprocal hybrid male sterility (whereby one male is sterile and the other is fertile, from a reciprocal cross) emerges to be a common pattern among closely related species when both reciprocal hybrids are assessed for fertility (Dobzhansky, 1934; Prakash, 1972; Bock, 1984). This pattern seems to hold true not only for whole chromosomal associations, found in F_1 hybrids, but for partial chromosomal segments as well. For example, Wu & Beckenbach (1983), introgressed certain X-linked regions of *D. persimilis* into a *D. pseudoobscura* background. They found that the reciprocal introgressions (introgressions of the same X-linked regions of *D. pseudoobscura* into a *D. persimilis* background) did not produce the same effects on fertility. These observations of nonreciprocal hybrid male sterility are a natural consequence of the two-locus model of reproductive isolation (Muller, 1942) as long as one of the loci involved is sex-linked (the other loci must be on a different chromosome).

If one extrapolates the formation of interactions between these reciprocal hybrids, *reciprocal* hybrid male sterility would be expected. The nature of the sterility phenotypes would, however, differ from each other since the new sterility interactions represent independent events. Such 'asymmetries' in the sterility phenotype were found between

reciprocal crosses of the *simulans* clade (Table 3.6) and hence, indicate the presence of independent substitutions of different spermatogenic loci. Thus, the early stages in speciation may be thought to take place through a number of steps. The genic asymmetry resulting from the two-locus model can directly result in the observational asymmetry of nonreciprocal hybrid male sterility. And the accumulation of at least two more substitutions can bring about reciprocal hybrid male sterility whereby the sterilities of each reciprocal hybrid are of different natures.

It should also be noted that both observational asymmetries (non-reciprocal hybrid male sterility and reciprocal hybrid male sterility with asymmetrical sterility phenotypes) are usually found in the heterogametic sex because of the presence of an asymmetrical platform in the form of an unbalanced chromosome set (see **1.7**)

4.7 Patterns of spermatogenic defects and phylogenetic inference

By observing trends of premeiotic vs. postmeiotic abnormalities in F_1 hybrids, inferences about the phylogenetic history of the parental species may be entertained. Assuming that the probability is higher that a novel mutation takes place in spermiogenesis rather than an earlier spermatogenic stage (Lindsley & Tokuyasu, 1980), it could be argued that *D. simulans* and *D. sechellia* have diverged most recently. Both hybrid genotypes produced from this species pair display postmeiotic defects. *D. mauritiana*, on the other hand, produces reciprocal hybrids conferring the two classes of sterilities, postmeiotic and premeiotic, when crossed to either *D. simulans* or *D. sechellia.*

Another perspective may be more effective. If we take into account the large effect

of the X-chromosome on hybrid male sterility (Coyne & Orr, 1989), as well as the various theoretical and empirical evidence on the interaction of the X-chromosome on either the Y-chromosome or the autosomes (see **1.5**), the relative effects of sterility incurred by the X-chromosome of different species may be evaluated. Under this scenario, the X-chromosome stemming from *D. mauritiana* seems to hold the largest effect on hybrid male sterility when hybridized to other species of this clade, since premeiotic lesions are produced in the hybrids. *D. sechellia* and *D. simulans* have smaller effects, relative to *D. mauritiana,* but more-or-less equal sterility effects when compared to each other. These results support either the faster divergence of spermatogenic loci on the X-chromosome of *D. mauritiana*, or a longer divergence time.

Coyne & Kreitman (1986) have similarly shown that there has been less divergence between *D. sechellia* and *D. simulans* than between *D. mauritiana* and *D. simulans* in reproductive characters. Such traits were comprised of both morphological and isolating characters. The morphological traits evaluated consisted of sex-comb teeth number, testes colour and genital arch shape. For the reproductive isolating character, they reported that the sterility in backcross F_2 males was less pronounced in *sec/sim* hybrids than in *mau/sim* hybrids. Such a difference in F_2 sterility profiles is congruous to the F_1 sterility profile of this study.

4.8 Directions for future research

Novel research usually brings about more questions than answers. This study is no exception. The understanding of differences in spermatogenesis, at the species level, is instrumental towards the understanding of hybrid male sterility and species formation. Testes substructure and the normal progression of spermatogenesis are obvious and important indicators of spermatogenic differences between species.. A large number of diverse strains from each species should be analyzed for such indicators, with the use of light microscopy, in the search of a species-specific pattern. The recent availability of testes specific P-element markers in members of this clade (Gonczy *et al.*, 1992; Castrillon *et al.*, 1993; True *et al.*, 1996; Davis *et al.*, 1996) will prove to be an invaluable tool in the characterization of such spermatogenic-specific traits. In addition, dissimilarities between species at the ultrastructural level have not been well characterized and may also be important in revealing the specific nature of sterility interactions in interspecific hybridizations.

In hybrids of the *simulans* clade, it was discovered that many of the incompatible interactions were manifested in spermiogenesis. Are there greater differences amongst homologs of these genes than other spermatogenic genes in the parental species? Or are these genes more sensitive to perturbation? An assay that may resolve such questions would involve a sampling of proteins from different levels of the testes, using 2D electrophoresis, corresponding to different stages of spermatogenesis. Such a sampling would be done in different species with various divergence times. And characterizations of spermatogenic defects in their hybrids may reveal which particular protein differences are prone to cause certain spermatogenic abnormalities. One major conclusion of this study was that each hybrid

genotype produced a characteristic sterility type. It was shown that the F_1 sterility of a particular hybrid genotype is not variable, but rather constant. This pattern holds true whether the comparison is made on spermatogenic defects between individual hybrids within strains, or between strains. But a single strain (*D. simulans-2119*) created an exception to this pattern. Thus, many more strains must be tested for this notion to be critically evaluated. A large substructural (midtestes cross-sections) or ultrastructural (electron micrographs) characterization would be too expensive an endeavour. However, a large-scale whole mount analysis (utilizing light microscopy on dissected testes) may prove to be a feasible and rewarding alternative.

The characterization of F_1 hybrid sterility reveals an assortment of incompatible interactions. Yet characteristics such as the degree of sterility differences between reciprocal hybrids and the overall differences between the stages of hybrid spermatogenic arrest may allow us to unfold the developmental progression of hybrid male sterility. Are certain interactions more prevalent than others at particular stages of speciation? Surveying such interactions, in a diverse array of hybrid male genotypes and correlating the resulting 'distributions of sterility' to such measurements as genetic distance as well as reproductive isolation indices may be an important aid in the understanding of the succession of hybrid male sterility.

The factor(s) found in the *D. simulans-2119* line is very interesting. This factor(s) seems to cause the reversion of the F_1 sterility defect from a postmeiotic one to a premeiotic one. First, does this factor affect the hybrid females? The fertility of F_1 females could be assessed and the possibility that this factor may cause a defect in a common reproductive trait

such as meiosis would be effectively evaluated. Secondly, is fertility reduced when this line is crossed to other strains of *D. simulans*? Intraspecific fertility between these strains and other *D simulans* strains produced individualized spermatids in the seminal vesicle, indicating fertility. However, an intraspecific midtestes sperm bundle assay will allow the trait of fertility to be effectively quantified. Thirdly, where does this factor(s) lie? The crossing scheme of Figure 2.2 could be reduced to only one generation of intraspecific hybridization, followed by the female progeny's interspecific cross to *D. sechellia* (3590). By observing testes of 1-2 day old progeny (greater than 3 day old testes will bring about ambiguous results because of *D. sechellia* (female) X *D. simulans* (male) sperm bundle degeneration), the proportion of individuals with postmeiotic cysts (sperm bundles) can be assessed. If monomorphic for these postmeiotic cysts, the factor(s) could be concluded to be on the Y-chromosome. However, if dimorphic (i.e. the presence of both post and premeiotic cyst in individuals of the sample), the factor(s) would be concluded to be located on one of the autosomes. This assay will further ascribe this interspecific interaction as either X^{sec}-Y^{2119} or X^{sec}-A^{2119}. The participation of modifier loci (loci of weak effect) may also be assessed.

And finally, the *complete* evaluation of cytoplasmic influences on hybrid male sterility has seemed to have reached an impasse with today's genetic tools. However, pole-cell transplantations may effectively solve such an inquiry, by fully evaluating the role of maternal effect factors.

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