# Conspecific Sperm Precedence in *Drosophila*

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# Abstract

Sexual selection is an important biological process that drives changes in reproductive traits. Characteristics that enhance a male's ability to generate progeny and contribute to the gene pool are favored under high selective pressure. Biologists have shown in numerous species that competition between sperm of different males can occur after mating. *Drosophila* females will remate in wild and laboratory populations creating opportunity for sperm competition. Normally the second male to mate produces the majority of progeny; however, conspecific sperm precedence is the phenomena whereby the male of the conspecific species fathers the majority of the progeny regardless of mating order. This form of postmating prezygotic isolation may serve as a barrier between closely related species promoting interspecies divergence and speciation. The aim of this project was to identify loci within the Drosophila genome that account for conspecific sperm precedence among species of the simulans clade (*D. simulans* and *D. mauritiana*). Using lines of *Drosophila simulans* with known genome segment introgressions from *D. mauritiana* I have identified specific introgressions that reduce the male's sperm competitive ability to values similar to those observed in crosses involving heterospecific males. The use of genome sequence data currently available in public databases allowed me to identify those candidate genes within the site of introgressions.

# Acknowledgments

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# Introduction

Sexual reproduction occurs in a wide range of organisms, from insects to mammals, and has evolved as a way for species to maximize their potential of survival. Due to the nature of sexual reproduction, mechanisms have evolved that allow for discriminating selection of favorable characteristics from generation to generation. Natural selection is an evolutionary process whereby the organisms most adapted to their environment contribute the majority of genes to the gene pool of the next generation. The Darwinian fitness of an organism is defined as the relative success of a genotype as measured by its ability to survive in the environment, productiveness in mating, and the ability to produce viable offspring (Freeman & Herron, 2001). Sexual selection is a form of selection that adequately explains the occurrence of sexual dimorphism within species and the presence of the elaborate secondary sexual physical characteristics that occur in nature (Freeman & Herron, 2001).

## Sexual Selection

Charles Darwin brought to light the idea of sexual selection and suggested that it is differential reproductive success based on an individual's ability to contend for and secure access to mates (as cited in Singh *et al.*, 2002). The result of failing to produce offspring is that the

individual's genes are not propagated in the gene pool. As a result a male's ultimate goal should be to maximize the number of eggs he fertilizes (Singh *et al.*, 2002). Mating and producing offspring are integral parts to the life cycle of sexually reproducing organisms; therefore, secondary sexual characteristics that make an individual a more efficient competitor are under high selective pressure by the process of sexual selection (Simmons, 2001). The occurrence of sexual selection can be explained by the asymmetry of maternal *versus* paternal investment that occurs in sexual reproduction. The personal energetic costs associated with producing viable gametes can be very high. Therefore, to avoid gamete wastage, organisms in nature have evolved mechanisms to ensure that reproductive energies are focused on matings with the highest probability of success (Snook & Markow, 2002). As a result of anisogamy (unequal maternal and paternal gamete size) and differential parental care, male and female investments in gametes are not equivalent. Males produce substantially more gametes than females as sperm are smaller and contain less protein and extracellular material than eggs (Simmons, 2001). As sperm are less costly to produce than eggs, a male could produce a very high quantity of gametes and is therefore limited in reproductive capacity only by his access to female mates (Bateman, 1948; Simmons, 2001). Female eggs are costly to produce; therefore, a female should be limited in reproductive capacity not by the number of mates she has access to but by the genotype of her

mate. As a result of this existing asymmetry we expect females to be the choosy sex and males to compete for access to females (Simmons, 2001). Sexual selection can exist in two forms. The sum of the interactions that occur between the male and female sex resulting in female choice is known as intersexual selection, whereas intrasexual selection is the competition between members of the same sex for preferential access to members of the opposite sex. Intrasexual selection can manifest itself in many ways, from the use of morphological secondary sexual traits in physical battles to more subtle competition between sperm.

## Sperm Competition

In recent years biologists have realized that sexual selection continues after copulation through forms of postmating prezygotic sexual selection. For a male, successfully securing a female and copulating does not guarantee reproductive success (Civetta & Singh 1999; Lawniczak & Begun, 2005). Sperm competition occurs after mating and before the formation of a zygote. It is defined as the competition within the female reproductive tract of sperm from two (or more) males for access to the female ova (Parker, 1970). Female *Drosophila* remate both in laboratory and wild populations (Harshman & Clark, 1998; Snook, 1998; Jones & Clark, 2003). Using specialized sperm storage organs, a pair of spermathecae and the seminal receptacles, a female is able to store the ejaculates from all previous matings for extended periods of time (Civetta,

1999). Stored sperm is used to fertilize eggs as they descend through the oviduct for the duration of the reproductive life of the female (Neubaum & Wolfner, 1999; Civetta, 1999). Given some of the fitness cost associated with mating (Chapman *et al.*, 1995; Civetta & Clark, 2000), it is not clear why females remate. A well-supported hypothesis is that female remating protects against male sub-fertility and allows the females to maximize her offspring's genotype (Singh *et al.*, 2002). The presence of sperm from more than one male in the female reproductive tract allows for sperm competition and generates the opportunity for post mating sexual selection in *Drosophila*. A male's fitness is greatly determined by his ability to compete with resident sperm or interfere with incoming sperm from subsequent matings (Price, 1997). When a female *Drosophila* doubly mates with conspecific males in the lab or in the wild the second male to mate is known to father the majority of the progeny, typically above 85%, ranging from 66 – 100% depending on species (Clark *et al.*, 1995; Jones & Clark, 2003). A conspecific male is defined as a male of the same species; whereas, a heterospecific male is defined as a male of a different but closely related species. Laboratory experiments that test for the occurrence of sperm competition in *Drosophila* use female double matings in which males with differing phenotypes compete against each other and paternity is determined on the basis of progeny physical characteristics (e.g. body color, eye color, etc). For experiments in the

wild, molecular markers are commonly used to fingerprint progeny as sired by different males (Harshman & Clark, 1998; Jones & Clark, 2003).

## Conspecific Sperm Precedence

The evolution of reproductive isolating mechanisms is key to speciation of closely related species (Fricke & Arnqvist, 2004). Research on reproductive barriers and speciation in internally fertilizing species has traditionally focused on events that occur before copulation and after the formation of a zygote (Price *et al.*, 2000). As a result of previous research, mechanisms such as behavioral mating discrimination, and gametic incompatibilities resulting in hybrid sterility and their evolutionary significance have long been appreciated as contributing factors to species isolation (Price *et al.*, 2000). In the absence of, or working together with other reproductive barriers, postmating prezygotic sexual selection may contribute greatly to the separation of populations into closely related species (Fricke & Arnqvist, 2003). Conspecific sperm precedence can drastically limit gene flow between closely related species both in laboratory and wild populations (Howard *et al.*, 1998). If a female is mated to both a conspecific and a heterospecific male, the conspecific male will father the majority of the progeny regardless of mating order (Price, 1997). This is known as conspecific sperm precedence (CSP) and it is one of the most notable postmating/prezygotic barriers to hybridization and gene flow (Price, 1997; Dixon *et al.*, 2003; Geyer &

Palumbi, 2004). Conspecific sperm precedence has been previously documented in flour beetles, crickets, grasshoppers, and plants (Hewitt *et al.*, 1989; Bella *et al.*,1992; Wade *et al.*, 1994; Gregory & Howard, 1997; Howard, 1999). The proposed mechanisms of conspecific sperm precedence between species vary greatly, are largely unproven, and are not currently attributed solely to sperm competition (Price, 1997). Conspecific sperm precedence is a type of reproductive isolation that is only observable after multiple matings have occurred. Using three *Drosophila* sibling species *D. simulans*, *D. mauritiana,* and *D. sechellia*, Price (1997) directly compared the number of hybrid progeny produced after a single heterospecific copulation with the number of hybrids produced after both a heterospecific and a conspecific copulation. By performing single conspecific and interspecific matings between *D. simulans* and *D. mauritiana* it was determined that the number of hybrid progeny produced was equivalent to or greater than the resulting progeny from a single conspecific mating. *D. simulans* females after doubly mating to both one *D. simulans* and one *D. mauritiana* males produced on average more than 85% pure-species progeny regardless of mating order of the males (Price, 2000). When the conspecific male was first to mate he fathered 86% of the progeny and 99% when second to mate (Price, 1997). Production of hybrid progeny was limited only when the female was mated to a conspecific male indicating reproductive isolation between species was caused by competition between their ejaculates.

This demonstrates the presence of conspecific sperm precedence and its action as a competition dependent phenomenon.

## Interspecies genetic introgressions

Dr. Yun Tao of Harvard University has generated strains of *D. simulans* flies with known introgressions of *D. mauritiana* DNA. The introgressions are limited to the third chromosome and are the only significant difference in genetic make-up between the introgressed males and pure *D. simulans* males (Tao *et al.*, 2003). Therefore, these strains can be used to quantify phenotypic differences and establish phenotype-genotype associations. We know that conspecific sperm precedence occurs between *D. simulans* and *D. mauritiana*, creating a drastic difference between species in phenotypic values. We can use the introgressed strains to test the effect of specific *D. mauritiana* DNA sequence insertions, into a *D. simulans* genomic background, on male's sperm competitive ability. These *Drosophila simulans – D. mauritiana* interspecies introgressed strains are better suited for phenotype-genotype association studies done in the past using *D. simulans – D. sechellia* introgression lines (Civetta *et al.*, 2002; Civetta & Cantor., 2003; Civetta *et al.*, 2005) because the mapping approach does not rely on the use of randomly spread anonymous molecular markers but on well mapped genome introgressions.

## Objective

The objective of my thesis is to map loci within the *Drosophila* genome that account for conspecific sperm precedence among species of the *simulans* clade (*D. simulans* and *D. mauritiana*). Using this information I shall identify candidate genes responsible for conspecific sperm precedence.

# Materials and Methods

## Fly stocks

A *Drosophila simulans* ebony stock used was obtained from Flybase, Tuscon (laboratory stock #14021-0251.033). Flies were homozygous for recessive ebony mutation located on the third chromosome conferring black body color. Dr. Yun Tao of Harvard University kindly provided sixty introgressed *Drosophila simulans/mauritiana* lines. Tao *et al.* (2003) generated these strains using the following two-stage protocol:

In phase one, highly inbred *D. simulans*, *sim 1 x 1 JJ*, females were crossed to *D. mauritiana* males with a P[w+] insert. P[w+] inserts are semi-dominant markers with various eye colors ranging from orange to red. Resulting orange-eyed female progeny were then backcrossed to *sim 1 x 1 JJ* for five generations (BC5). Orange eyed BC5 males were then crossed to *sim*B females that contained visible markers on the X and second chromosomes. Using visible (phenotypic) and allele-specific oligonucleotide (ASO) markers as guides, strains for each P[w+] insert were selected on the basis of their common X and second chromosome *D. simulans* genetic background.

In phase two the introgressions of *D. mauritiana* DNA within the sublines were made shorter. Orange eyed males of the introgressed strains were mated to *sim*B females generating recombinant flies. New

strains with shorter introgressions were established and then identified by genotyping males with appropriate ASO markers (Tao *et al.*, 2003).

The 60 introgressed lines used in my thesis are a subset of those created by Dr. Tao (Figure 1), chosen on the basis that each carried different *D. mauritiana* introgressions and that they collectively spanned the entire third chromosome.

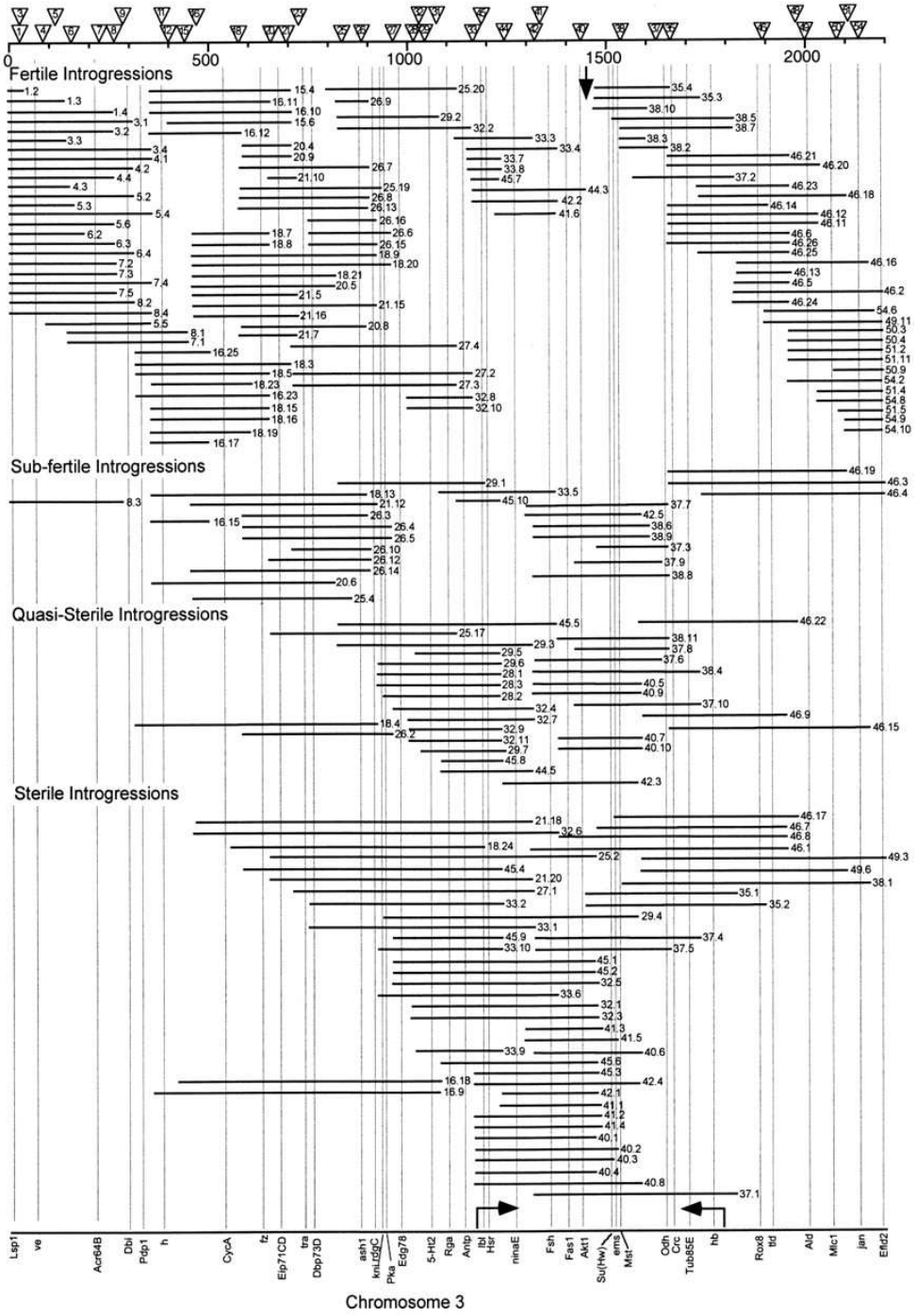



**Figure 1**: Bars represent introgressions of *D. mauritiana* DNA on an otherwise pure *D. simulans* background. Numbers represent the lines to which the introgressions belong, with digits before the dots referring to P[w+]-inserts (triangles at top). Cytological positions are based on a *D. melanogaster* map; note bent arrows indicate an inversion between *D. melanogaster* and *D. simulans*. Introgressions are grouped based on fertility of homozygous males (Tao *et al.*, 2003).

## Stock Maintenance

Stocks were maintained at room temperature on 9-hour light and 15 hour dim cycles. Stocks were kept in vials and then expanded into bottles to produce sufficient numbers of progeny for sperm competition assays. Introgressed fly stocks were maintained using the following protocol:

Introgressed lines were anesthetized with $CO_2$ gas, males carrying introgressions were selected based on the presence of orange eyes resulting from the P[w+] insert, the remaining flies were discarded. In every generation, stocks for each introgressed strain were initiated by crossing orange-eyed males with *sim*B females. Parental flies were left to mate for seven days at the end of which time they were removed and dental rolls were inserted into the food to maximize the recovery of adults as the rolls help larvae pupate away from the liquid medium.

All other stocks used were maintained in a similar fashion. Parental flies were collected and placed into new bottles, left to mate for seven days and then dumped. Progeny begin to eclose 14 days after the beginning of oviposition.

## Media

Flies were raised on a standard cornmeal molasses medium (Table 1). The media was heated and prepared in a stainless steel pot on a hotplate. Upon completion of cooking the media was dispensed using a

peristaltic pump into autoclaved reusable plastic vials or bottles. Disposable vials were used during matings due to increased clarity of the plastic.

**Table 1**: Standard Cornmeal-molasses media recipe for *Drosophila*. Combine cornmeal, yeast, and agar; mix with cold water to make a slurry. Add to rapidly boiling water and stir continuously until it comes to a second boil. Remove from heat add molasses. Cool in water bath to 65°C then add Tegosept and proprionic acid. Dispense into vials or bottles before it cools further. One set yields 100 vials or 18 bottles.

| Ingredient | Quantity |
|---|---|
| Cornmeal | 65 g |
| Brewers Yeast | 13 g |
| Agar | 6.5 g |
| Cold Water | 170 mL |
| Boiling Water | 760 mL |
| Molasses | 45.5 mL |
| 99% Proprionic acid | 5 mL |
| 10% Tegosept* | 20 mL |

* 50 g methyl-hydroxybenzoate per 500 mL 95% ethanol

## Virgin Fly Collections

New sets of flies were initiated (placed) into bottles containing fresh medium. Male and female flies were allowed to breed and oviposit for seven days. At the end of seven days parental flies were removed and

discarded. Dental rolls were inserted into the media (to aid in eclosion of larvae) in bottles containing introgressed strains of flies. On the first day of collection, any flies present in the bottles were dumped. Flies were then collected from the *D. simulans (e/e)* stocks on a 5-hour cycle to ensure virginity of females. Females will remain virgin for up to 8 hours at 24°C (Greenspan, 1997). Flies were anesthetized using $CO_2$ gas and sex was determined based on observation of genitalia using a light-dissecting microscope. Collections were carried out three times a day at 9 am, 2 pm and 7 pm. Females and males were collected and stored separately (until initial matings) in new vials of 10 and 20 respectively.

## Sperm Competition Trials

Introgressed *Drosophila simulans/mauritiana* males from 16 different lines were used in double-mating experiments to test for sperm competitive ability. Virgin 2-4 day old *Drosophila simulans* females homozygous for the *ebony* (*e/e*) mutation (black body coloration) were mated to 2-4 day old *D. simulans* (*e/e*) males. Double matings were carried out according to the following standard protocol (Civetta *et al.*, 2002):

1. Initial matings were *en masse* for a period of two hours in a vial containing 10 females and 20 males. Females were then individually aspirated to separate vials (vial 1) and males were discarded.

2. Two days later, females were presented with one or two males from each of the 16 introgressed lines. Matings were observed every 15 minutes (copula last 20 to 30 minutes) and recorded for a period of 8 hours. Vials, where no mating was observed, were left overnight. In the morning males were discarded and females were aspirated individually into new vials (vial 2).
3. Four days later each female was individually transferred to vial 3.

Vial 1 was used to confirm the initial mating based on the presence of progeny. Progeny from vials 2 and 3 were scored based on phenotypic body coloration that allowed for determination of paternity. Counts for vials 1, 2, and 3 were taken on the $20^{th}$, $25^{th}$, and $24^{th}$ days after the beginning of ovipositioning in each vial respectively. Only females that produced both ebony and wild type progeny were considered for data analysis.

## Data Analysis

Statistical analysis consisted of a one-way analysis of variance (ANOVA) performed using the SPSS statistic package (version 11.0). ANOVA was used to test for statistically significant difference among introgressed lines in the proportion of progeny sired by the second male to mate (P2 values -Boorman & Parker, 1976). P2 values were

transformed using the arcsine transformation (angular transformation) to fulfill the requirements of normality (Sokal & Rohlf, 1995).

## Identification of Candidate genes

### Flybase

Candidate genes were identified using the genetic map of *D. melanogaster* available at Flybase (http://flybase.bio.indiana.edu/). Candidate genes were selected based on their location, a section of the third chromosome that showed significant effects on sperm competitive ability. I narrowed down the list by focusing on genes expressed in male reproductive tissue.

Tissue of gene expression was determined using a gene expression search via term link available at Flybase. Termlink categorizes genes by anatomy followed by organ systems. Within organ system it was possible to narrow down the search to the male reproductive system. A search for all genes within the male reproductive system was carried out yielding 571 known genes. Candidate genes were then narrowed down by chromosome map position. The DNA sequence of candidate genes was retrieved from links to the *D. melanogaster* genome scaffolds (available at GenBank) and saved in FASTA format, DNA sequence information on the *D. simulans* and *D. sechellia* gene orthologs (genes similar as a result of a common ancestor) to our *D. melanogaster* candidate genes was obtained using the following protocol:

1) Search for gene at FlyBase (http://flybase.bio.indiana.edu/) using its full name or abbreviation and searching within "any field"
2) Click gene symbol and click scaffold (or gene) accession number
3) Under Edit, use "Find (On this page)" and type the name/ abbreviation of the gene
4) Click "gene" on the left
5) Under "Display", choose FASTA and "show" TEXT.
6) Save as text with filename: "Dmel_genename.txt"
7) Click BLAST from the GenBank page (http://www.ncbi…) and select "insects" within the option "Genomes"
8) Paste query gene sequence (the Dmel_genename.txt file) and select search *D. simulans* and *D. sechellia* genomes.
9) Record "hits" to the *D. simulans* and *D. sechellia* genomes

## Blast

Performing BLAST (basic local alignment search tool) consists of aligning the query gene sequence from *D. melanogaster* to the partial genome reads of *D. simulans* and *D. sechellia*. As a result of the blast searches percentage identity, expected (E) values, and score bits were generated. Percentage identity or sequence similarity defines how alike sequences of nucleotides coding for the same gene are. Percentage gap is also taken into consideration. A gap is an insertion and/or deletion of nucleotides in one sequence of DNA relative to another. Gap scores are generated to prevent the buildup of too many gaps within a single alignment. This is because alignment algorithms work by maximizing the number of similarities among sequences and the introduction of gaps

cause the subtraction of a fixed amount from the score of percentage identity (www.ncbi.nlm.nih.gov/blast). The E value is a parameter that describes the number of nucleotide matches between genomes expected as a result of random chance. As the E value approaches zero, sequences are considered significantly similar. Score Bits refer to the relative length of the query gene sequence that "hit" or matched with sequences from the partial *D. simulans* and *D. sechellia* genomes. Due to potential overlap of random nucleotide sequence reads in *D. simulans* and *D. sechellia*, average E values were reported when there was more than one hit to the *D. melanogaster* query sequence. The relationship of E values to P values is as follows:

$$P=1-e^{-E}$$

However, when E < 0.01 the two values are nearly identical and P values and E values may be used interchangeably (www.ncbi.nlm.nih.gov/blast).

# Results

A total of 517 *D. simulans* (e/e) females were set up to doubly mate with *D. simulans* (e/e) male and an introgressed male. Two hundred and twenty one females were doubly-mated to both a *D. simulans* and an introgressed male. P2 values, proportion of progeny sired by the second male to mate in a double mating, were obtained only from the females who doubly mated. Angular transformation of P2 proportions adequately fit the assumptions of homoscedasticity and normality satisfying the requirements to carry out analysis of variance (ANOVA). Transformed data was used in ANOVA. The mean P2 value of all lines was 0.57 (Table 2). However, significant variation in sperm competitive ability was seen between the lines ($F= 6.33$; $P<0.0001$), (Table 3).

**Table 2**: Average P2 results for progeny from 221 females doubly mated to both *D. simulans* and males from the 16 introgressed *simulans*/*mauritiana* lines. N represents the total number of females that double mated. SD is standard deviation and SE is standard error.

| **Line** | **Mean** | **N** | **SD** | **SE** |
|---|---|---|---|---|
| 16.3 | .690 | 21 | .275 | .060 |
| 16.9 | .679 | 25 | .251 | .050 |
| 21.12 | .681 | 24 | .181 | .037 |
| 27.2 | .812 | 19 | .165 | .038 |
| 25.17 | .694 | 7 | .225 | .085 |
| 32.1 | .172 | 4 | .071 | .036 |
| 32.4 | .294 | 17 | .280 | .068 |
| 32.6 | .460 | 17 | .305 | .074 |
| 32.7 | .270 | 7 | .333 | .126 |
| 32.10 | .698 | 11 | .274 | .083 |
| 33.3 | .678 | 6 | .296 | .121 |
| 33.4 | .455 | 14 | .353 | .094 |
| 33.5 | .352 | 12 | .196 | .057 |
| 45.6 | .369 | 14 | .317 | .085 |
| 45.8 | .643 | 11 | .254 | .077 |
| 45.9 | .661 | 12 | .247 | .071 |
| Total | .569 | 221 | .305 | .021 |

**Table 3**: Summary of ANOVA values testing for variation in P2 proportions between 16 introgressed *simulans*/*mauritiana* lines competed against *D. simulans* males.

| Source | df | MS | F |
|---|---|---|---|
| Line | 15 | .643 | 6.33*** |
| Error | 205 | .102 | |

*** P < 0.001

A P2 value of 0.5 represents equal usage of both conspecific and heterospecific sperm and would be the value expected as a result of free sperm mixing within female's sperm storage organs. Values significantly lower than 0.5 are indicative of conspecific sperm advantage. Analysis of the data revealed five strains of introgressed males showed P2 values significantly lower than 0.5 (Figure 2). Using a map available from polytene chromosome studies in *Drosophila melanogaster*, it was possible to determine a region of the chromosome responsible for the lack of sperm competitive ability observed in the five strains. However, it is known that *D. simulans* and *D. mauritiana* chromosomes differ from *D. melanogaster* in that they share a chromosome inversion on the 84F to 93F polytene bands in the third chromosome (Lemeunier & Ashburner, 1976). The introgressions in the *simulans/ mauritiana* strains showing evidence of poor sperm competitive ability overlap in a region on the third chromosome containing the chromosome inversion. The equivalent map position in *D. melanogaster* is 78CD to 84F; 93F to 88B. A search for *D. melanogaster* genes located between 78CD- 84F; 88B- 93F was conducted on Flybase (http://flybase.bio.indiana.edu/). Candidate genes were selected based on their location on the chromosome, their known effect on phenotype and the tissues in which they are expressed. A total of 15 candidate genes were identified on Flybase in *D. melanogaster* (Table 4). Within *D. simulans* a study by Swanson *et al.* (2001) allowed for identification of 14 accessory gland EST's (expressed sequence tags)

within *D. melanogaster* located between 78CD- 84F; 88B- 93F (Table 5). In total 29 candidate genes were identified within the region of interest in the *D. melanogaster* genome (Figure 3).

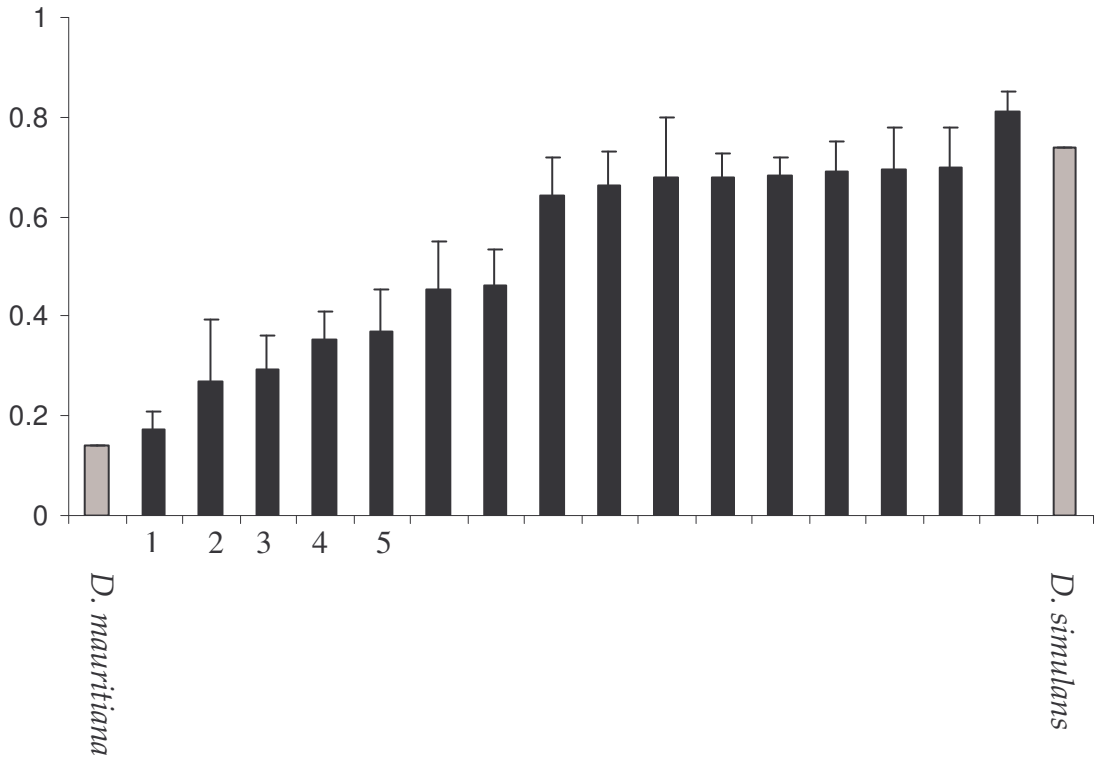



**Figure 2**: Mean (+SE) number of hybrid progeny (P2) produced after a double mating of *D. simulans* females to males from 16 introgressed strains. Parental values for *D. mauritiana* and *D. simulans* are indicated on either end of the graph (from Price, 1997). Lines numbered 1 to 5 are those showing P2 lower than 0.5.

**Table 4**: Results for candidate genes located on the third chromosome of *D. melanogaster* within region of interest 78CD-84F; 88B-93F. % I indicates percentage identity, percentage of alignment gap are in parenthesis, and E represents E-value. Results generated by alignment against partial genome reads of *D. simulans* (*sim*) and *D. sechellia* (*sec*).

| **Gene** | **Symbol** | **Location** | **Accession number** | **% I (*sim*)** | **% I (*sec*)** | **E (sim)** | **E (sec)** |
|---|---|---|---|---|---|---|---|
| chromosome bows | *chb* | 78C1-2 | AE003593 | 96% (0) | 96% (1%) | 0.0 | 5e-94 |
| Spermatocyte arrest | *sa* | 78D1 | AE003594 | 93% (0) | 93% (0) | 0.0 | 0.0 |
| shank | *shk* | 82C1-5 | n/a | n/a | n/a | n/a | n/a |
| ripped pocket | *rpk* | 82C5 | AE003605 | 93% (1%) | 93% (1%) | 0.0 | 0.0 |
| bobble | *bob* | 82D3-8 | n/a | n/a | n/a | n/a | n/a |
| meiosis 1 arrest | *mia* | 83B1 | AE003602 | 92% (1%) | 91% (1%) | 0.0 | 0.0 |
| α-Tubulin at 84B | *aTub84B* | 84B2 | AE003672 | 95% (1%) | 95% (2%) | 0.0 | 0.0 |
| don juan | *dj* | 84B2 | AE003673 | 94% (0) | 94% (0) | 5e-51 | 1e-168 |
| Glucose dehydrogenase | *Gld* | 84D3 | AE003679 | 91% (4%) | 95% (0) | 1.14e-5 | 2e-5 |
| rotund | *rn* | 84D3 | AE003672 | 92% (4%) | 93% (3%) | n/a | n/a |
| doublesex | *dsx* | 84E1-2 | AE003676 | 90% (4%) | 90% (4%) | 1.25e-4 | 5.08e-4 |
| puckered | *puc* | 84E12-13 | AE003677 | 89% (5%) | 94% (2%) | 0.0 | 0.0 |
| Mst89B | *Mst89B* | 89B | AE003712 | 89% (3%) | 79% (6%) | 0.0 | 0.0 |
| Maternal transcript 89Bb | *Mat89Bb* | 89B9 | AE003712 | 97% (0) | 96% (0) | 0.0 | 0.0 |
| Rhodopsin 2 | *Rh2* | 91D4 | AE003724 | 96% (0) | 92% (2%) | 0.0 | 0.0 |

**Table 5:** Percent identity of candidate genes located on the 3rd chromosome of *D. melanogaster* genome within region of interest between 78CD-84F; 88B-93F (Swanson *et al.*, 2001). % I indicates percentage identity, percentage of alignment gap are in parenthesis, and E represents E-value. Results generated by alignment against partial genome reads of *D. simulans* (*sim*) and *D. sechellia* (*sec*).

| **Accession Number** | **Gene number** | **Location** | **% I *(sim)*** | **% I *(sec)*** | **E (*sim*)** | **E (*sec*)** |
|---|---|---|---|---|---|---|
| BG642337 | CG7139 | 79C2 | 92% (1%) | 92% (2%) | 0 | 0 |
| BG642236 | CG14560 | 79C3 | n/a | n/a | n/a | n/a |
| BG642306 | CG14560 | 79D | n/a | n/a | n/a | n/a |
| BG642201 | CG5837 | 79E2 | n/a | n/a | n/a | n/a |
| BG642142 | CG9769 | 82D6 | 97%(0) | 96% (0) | 0 | 0 |
| BG642232 | CG10284 | 83E6 | 91% (2%) | 91%(2%) | 0 | 0 |
| BG642137 | CG1965 | 84C1 | 95% (0) | 94% (1%) | 0 | 0 |
| BG642335 | CG2640 | 84C4 | n/a | n/a | n/a | n/a |
| BG642249 | CG5516 | 89A8 | 95% (0) | 95% (0) | 0 | 0 |
| BG642293 | CG14879 | 89B | 92% (3%) | 92% (3%) | 0 | 0 |
| BG642283 | CG4261 | 89B3 | n/a | n/a | n/a | n/a |
| BG642311 | CG8925 | 89B12 | 91% (4%) | 94% (1%) | 0 | 0 |
| BG642248 | CG5862 | 93D1 | 96% (0) | 95% (0) | 8e-47 | 0 |
| BG642295 | CG17843 | 93F10 | 95% (0%) | 94% (0%) | 0 | 0 |

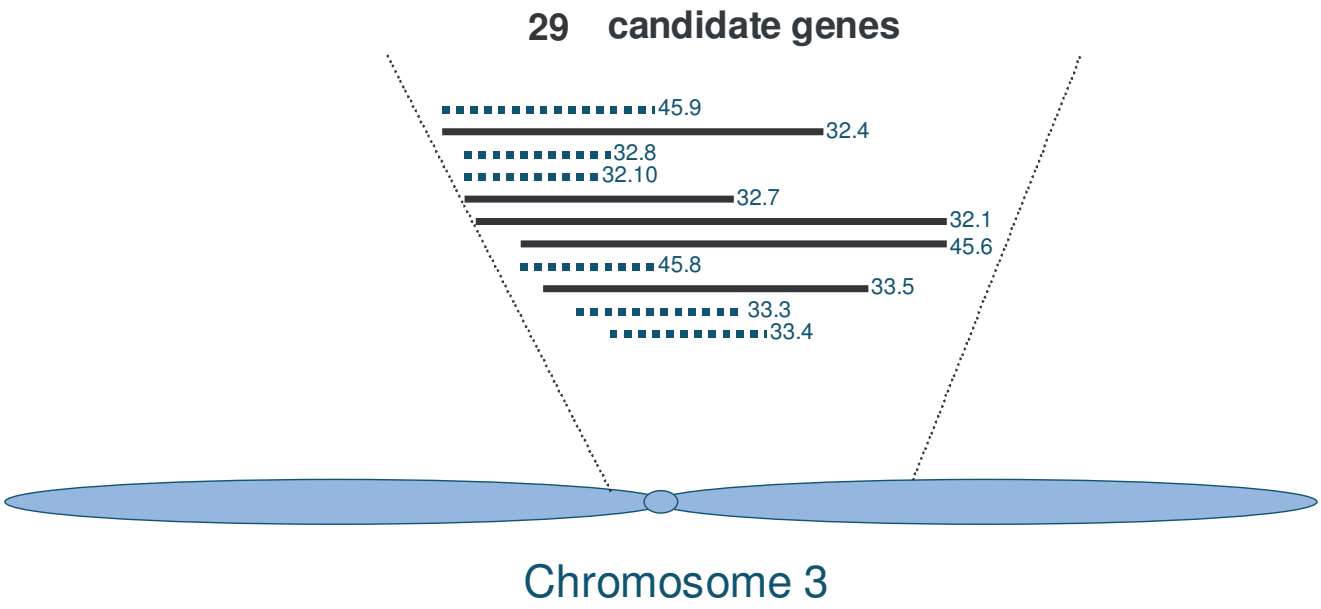



**Figure 3**: Diagrammatic representation of eleven introgressions between 78CD- 84F; 88B- 93F in Chromosome 3 of *Drosophila*. Solid bars represent introgressions within the chromosome that showed P2 <0.5 indicating a gene(s) for conspecific sperm precedence is present. Dashed bars indicate introgressions where P2 >0.5.

## E values

E values for gene sequences from the *D. melanogaster* genome aligned against *D. simulans* and *D. sechellia* were similar. E values equal to zero indicate gene sequences are identical and are highly conserved between species within the clade. For eleven genes scaffold (sequence) were not available on Flybase; therefore, it was not possible to perform BLAST. Results for the 21 candidate genes, where scaffold information was available, from (Table 4 and Table 5 ) are summarized below:

| *D. melanogaster* Aligned against | E-value >0.0 | E-value = 0.0 | Percent Identity ≤ 90% |
|---|---|---|---|
| *D. simulans* | 4 | 17 | 3 |
| *D. sechellia* | 4 | 17 | 2 |

Genes with E values greater than zero represent good potential candidate genes. *Chromosome bows*, *don juan*, *glucose dehydrogenase*, *double sex,* and BG642248 all had E values greater than zero.

## Percentage identities

An arbitrary percentage identity value of 90% was selected as the cut off point for good potential candidates. As a result, 3 genes were selected as good potential candidates *Mst89B*, *doublesex*, and *puckered*.

Of these genes, only *doublesex* was identified as a good potential candidate by both percentage identity and E value.

# Discussion

When female *Drosophila* is doubly mated with both a conspecific and a heterospecific male, the conspecific male fathers the majority of progeny regardless of mating order. This phenomenon is known as conspecific sperm precedence (CSP). My results showed significant variation in sperm competitive ability among sixteen introgressed *simulans/ mauritiana* lines that were competed against *D. simulans* males in mating to *D. simulans* females. An introgression of *D. mauritiana* DNA into the third chromosome represented the only significant difference in genetic makeup among the introgressed males. Five of the sixteen introgressed lines produced less than 50% of the progeny output when made to compete against *D. simulans*, which indicates the *D. simulans* males are enjoying conspecific sperm precedence. Therefore, differences in sperm competitive ability among males from the introgressed *simulans/mauritiana* lines, with some males behaving as *D. mauritiana*, can be attributed to genetic differences in the third chromosome.

To ensure that differential progeny production is a result of postmating prezygotic barriers to fertilization, sub-par egg hatchability and larval inferiority needs to be ruled out. Price *et al.* (2000) placed known numbers of both *D. simulans* and hybrid *simulans/ mauritiana* fertilized eggs together into vials containing media, then Price *et al.* compared progeny output of both pure *D. simulans* and hybrid progeny

by measuring egg hatchability and larval competition. They reported no significant differences in either egg hatchability or larval competitive ability (Price *et al.*, 2000). Consequently, it was determined differential paternity success was not a consequence of differences in embryo viability and development of larvae. Therefore, differences in number of progeny sired by a conspecific and a heterospecific male in competition are not the result of differences in embryo development and viability but a consequence of conspecific sperm precedence. The study by Price *et al.* (2000) demonstrates that conspecific sperm precedence occurs within the simulans clade (*D. simulans*, *D. mauritiana* and *D. sechellia*) and that it plays an important role in restricting gene flow between species.

Conspecific sperm precedence has been well documented in a wide variety of species. However, to date the mechanisms by which conspecific sperm precedence occurs remain largely unknown. In recent years, research has focused on determining the cytolocation and identity of genes responsible for conspecific sperm precedence and other hybrid incompatibilities.

QTL (quantitative trait loci) mapping has been previously employed by Civetta *et al.* (2002) to determine the genetic basis for conspecific sperm precedence between *D. simulans* and *D. sechellia*. QTL involves testing associations between differences (alleles) scored at random molecular markers within the genome and phenotypic differences. The likelihood that a locus, present between two molecular markers, is

responsible for phenotypic differences is then calculated (Civetta *et al.*, 2002). A region of the third chromosome of *D. melanogaster* spanning from 69D- 77A was determined to be responsible for interspecific differences in male fecundity (measured as total number of progeny produced by females) between *D. simulans* and *D. sechellia* (Civetta *et al.* 2002). They determined a cytolocation for gene(s) responsible for interspecific differences in reproductive traits which is in close proximity to the region I determined to be responsible for conspecific sperm precedence between *D. simulans* and *D. mauritiana*, which is 78CD-84F; 88B-93F. These regions are both located on the third chromosome; however, for the most part they occupy different arms of the third chromosome and do not overlap. Though QTL mapping is useful, it is time consuming and expensive, as it involves assaying a large number of molecular markers to create dense chromosome maps and relies heavily on statistical inference. The advantage of using the introgressed flies generated by Tao (2003) in this experiment was the relative ease with which observed changes in phenotype could be attributed to known changes in DNA.

One result of competing introgressed males against *D. simulans* males was that overlapping introgressions within the same region of the third chromosome indicated both the presence and absence of conspecific sperm precedence. I have determined that conspecific sperm precedence is dependent on the size of the introgression and likely a

result of multiple gene effects, as introgressions long enough to make males poor sperm competitors (P2< 0.5) are likely to contain the full compliment of genes required for conspecific sperm precedence. Civetta *et al.* (2002) also concluded that the genetic basis of interspecific differences in reproductive traits are likely a result of complex genetic interactions involving multiple epistatic genes. Mapping can be difficult when multiple gene interactions are present. Excellent demonstrations of the involvement of complex epistatic interactions in interspecific differences and the challenge in mapping can be seen in attempts to map genes responsible for hybrid male sterility.

Historically two schools of thought on reproductive isolation between species have existed. Some biologist have attributed reproductive isolation to additive effects of multiple loci whereas others believe it could be attributable to a small number of major effect genes (Perez & Wu, 1995). In order to determine the genetic basis of reproductive isolation between closely related species, earlier experiments have employed the use of introgressions to study the hybrid male sterility (HMS) that results from interspecifc matings between *D. mauritiana* and *D. simulans* (Perez *et al.*, 1993). Perez *et al.* (1993) conducted an experiment in which numerous small segments of *D. mauritiana* DNA from the X chromosome were introgressed into a *D. simulans* background. Introgressions were monitored using a series of molecular markers and phenotypic mutations. After 20 generations of

backcrosses the introgressions were mapped using RFLP (restriction fragment length polymorphism) analysis; however, terminal boundaries of some introgressions were unknown as they passed the molecular markers used in the experiment. As a result of this experiment, a gene *Odysseus* (*Ods*) was determined to be a major factor contributing to HMS. Further molecular analysis revealed that *ods* is involved in HMS; however, it requires cointrogression of surrounding genes to confer complete HMS (Perez & Wu, 1995). Investigations into hybrid interactions have revealed that the majority of loci did not have large effects; however, larger effects were seen when more than one locus was present in an introgression (Tao *et al.*, 2003). The majority of genetic incompatibilities that manifest in species hybrids are recessive and epistatic (Presgraves, 2003).

The introgression approach is a useful tool to uncover the presence of genes of interest. Nonetheless, as with most techniques, its results must be appropriately analyzed and considered before assumptions are made. When working with introgressions as a tool for genetic mapping it is desirable to have the effects of the introgressed DNA manifest only in the phenotype being evaluated, as this limits confounding effects and allows for proper identification of gene function. However, this is generally not the case as the majority of genes are epistatic, meaning that they interact with and modify other genes at different loci within the genome. When DNA is introgressed from one genome into another, its

environment, and thus the environment of the genes, is changed. A novel environment for a gene may alter its normal function, either as a result of interactions with new genes, or a failure to interact with or be regulated by genes in its normal environment, thus resulting in incompatibilities (Perez & Wu, 1993; Civetta *et al.*, 2002, Tao *et al.*, 2003). For example, a gene with a small positive effect in its normal genome may have a large negative effect under hybrid conditions. Therefore, analyzing the effects and behavior of genes under introgressed conditions may not be representative of the true normal behavior or function of genes. By using introgressions of varying lengths that overlap within the same chromosomal region I was able to determine gene behavior in a number of hybrid environments.

Though candidate genes were identified using both percentage identity scores and E values, only those genes identified by percentage identity will be discussed. Percentage identity scores generated by comparing gene DNA sequences between *D. melanogaster*, *D. simulans* and *D. sechellia* represent a comprehensive evaluation of candidate genes, as percent identity takes into account the length of the sequences that align. Using an arbitrary 90% percentage identity cut off for good potential candidate genes *doublesex*, *Mst89B*, and *puckered* were identified. I will briefly discuss the known roles of each gene within *Drosophila*.

*Doublesex (dsx)* is essential in sex determination in *Drosophila*; its activity within reproductive anatomy makes *dsx* an excellent preliminary candidate gene. Sex determination in *Drosophila* involves a primary signal, a key gene, and a double switch. Proper *dsx* function is necessary for all somatic sexual development in males and females (Garrett-Engele *et al.*, 2002). Chromosomes, XY or XX, provide the initial signal resulting in repression or activation of the key gene *sexlethal* (*Sxl*). In males, *Sxl* is not transcribed and therefore no protein product is produced (Schutt & Nothiger, 2000). This causes *transformer* (*tra*) to be functionally off, due to sex specific splicing of the pre mRNA intron that reduces the open reading frame resulting in a shortened inactive *tra* polypeptide (O'Neil & Belote, 1992). This leads to splicing of *dsx* to the male form (*dsxM*). In summary the gene *dsx* has dual functions, repressing femaleness in males and repressing maleness in females (O'Neil & Belote, 1992).

Molecular evidence has shown the gene *doublesex* is highly conserved within the *D. melanogaster* subgroup. However, across Dipteran lineages variable levels of selective contraints on the male specific segment of the *dsx* protein indicate uneven selection pressures across taxa (Kulathinal, 2004). Sex determining gene cascades in Dipterans are shown to evolve more rapidly than other gene regulatory pathways, providing support for *dsx* as a candidate gene (Schutt & Nothiger, 2000; Kulathinal & Singh, 2004).

Unfortunately, little information exists on gene *Mst89B*. It is a testis specific expressed gene that was identified in *D. melanogaster.* Transcripts for *Mst89B* were found in germline-derived cells during spermatogenesis in *D. melanogaster* (Stebbings *et al.*, 1998). It is likely that *Mst89B* also represents a good potential candidate gene due to its expression during spermatogenesis.

The role of *puckered* (*puc*) in connection to reproductive development in *Drosophila* is largely unknown. However, some mutations in *puc* are known to affect the ejaculatory bulb, ejaculatory tract and male terminalia (http://flybase.bio.indiana.edu). *Puckered* is most well understood for its role as a key factor that prevents apoptosis in epithelial cells by restraining basal JNK signaling (McEwan & Peifer, 2005).

## Conclusion

By using a double mating approach it was possible to determine a region in the third chromosome that contains genes responsible for conspecific sperm precedence. By generating a list of candidate genes and BLASTing them against both *D. simulans* and *D. sechellia* I was able to determine, based on E values and similarity scores, three candidate genes for future studies.

## Future Directions

In the future, I plan to complete sperm competition trials in the remaining introgressed lines and to duplicate trials of those already tested. Using the same protocol, I plan to generate a more detailed list of potential candidate genes. Candidate genes that show dissimilarity between *D. simulans*, *D. sechellia* and *D. melanogaster* will be sequenced in *D. mauritiana* and used to perform comparative gene expression analysis between *D. simulans* and introgressed males from lines showing evidence of CSP.

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