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Characterization of the Immune Deficiency Pathway during female meiosis in Drosophila melanogaster

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Characterization of the Immune Deficiency Pathway during female meiosis in *Drosophila melanogaster*

A Thesis presented in partial fulfilment of the requirements for the degree of Master of Science

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Acknowledgements

Completing a thesis is no easy task and at the time I started in the master's program at DePaul was not the most ideal time, as this was in 2020. I want to first thank my advisor Dr. William Gilliland for his support throughout the whole process, especially since I came into the lab with very minimal knowledge when it came to *Drosophila melanogaster*. I would like to also thank my committee members Dr. Joanna Brooke and Dr. Philip Funk for giving feedback on drafts and for teaching interesting classes, as well as other faculty in the department. I'd also like to thank my parents for supporting me the best that could, even when they were entirely unfamiliar with what I was explaining, especially my mom. My cohort that I started this journey with was influential in helping me get through it even when I was struggling.

Abstract

Organisms can defend against pathogens by significantly increasing the diversity of their progeny, so that some progeny are more likely to survive infection. This led to the prediction that infection should cause an increase in recombination rates, which was previously shown by a study where female *Drosophila melanogaster* that were infected with the gram-negative bacteria *Providencia rettgeri* had increased rates of recombinant progeny (Singh et al. 2015). However, the mechanism that regulates recombination rates in response to infection is not understood and in that study they did not provide a mechanism for how that can occur. In an RNAi screen, our lab found the gene *mustard (mtd)* is required for normal recombination rates. This gene was previously shown to be part of the Immune Deficiency Pathway, helping protect flies against infection by *Vibrio cholerae,* another gram-negative bacteria (Wang, Berkey, and Watnick 2012). When *mtd* was knocked down by RNAi, we saw an ~95% reduction in recombination rates. This suggests that the IMD pathway may be part of the mechanism that modulates recombination rates in response to bacterial infection. We tested this by measuring if the recombination rate changes caused by *P. rettgeri* infection depends on *mtd* function. We found that infecting female flies with the bacteria did not have any change in recombination rates between the control groups and the experimental groups.

Introduction

Recombination is the process where chromosomes exchange material between homologous chromosomes during meiosis. Recombination was first discovered in the model organism *Drosophila melanogaster,* in Thomas Morgan's lab in the early 1900's (Sturtevant, 1913). *Drosophila melanogaster* has continued to be used to study recombination, which has revealed that organisms must regulate their recombination rates, as stressful events such as starvation, high temperatures and aging can change the proportion of recombinant offspring (Grell, 1978; Neel 1941) These events that change the recombination rate would have to occur early in meiosis during Prophase I, as this is when crossing-over occurs (Stevison et al. 2017).

Proper regulation of crossing-over is important because of two main reasons. First, recombination creates chiasmata, which lock the homologs together so that they can properly co-segregate during meiosis. Second, it shuffles the genetic information to diversify the progeny. This diversification is beneficial to individuals, because increasing the genetic variability of your offspring increases the likelihood that some offspring will have genotypes that are able to resist infection by pathogens, this has been observed in female *Aedes aegypti* that were infected with a parasite (Zilio et al. 2018). This has led to the prediction that active infections should cause recombination rates to increase.

This prediction that recombination rates should increase in response to infection was confirmed in a study which showed that female *Drosophila melanogaster* that were infected with the gram-negative bacteria *Providencia rettgeri* had increased recombination rates when compared to wounding-only controls (Singh et al. 2015). The recombination studies were only conducted in female flies, as meiotic recombination does not occur in male *Drosophila* (Stevison

et al. 2017). However, that study did not provide any mechanism for how bacterial infection could increase recombination rates.

A candidate mechanism for how recombination rates might be regulated came from an RNAi screen conducted by the Gilliland lab. This screen was to identify genes that cause defects during chromosome congression, the prometaphase process where chromosomes move out on the meiotic spindle and then return to form a well-structured single mass (Gilliland et al., 2009). One of the hits from this screen was the gene *mustard* (*mtd*). RNAi knockdown of this gene was found to result in 100% congression failure and 40% nondisjunction rates. This was the first meiotic phenotype known for *mustard*, an essential gene that results in the death of the fly if knocked out completely (Stowers, Russell, and Garza 1999).

Previously, *mtd* was shown to be part of the Immune Deficiency (IMD) pathway, which primarily defends against gram-negative bacteria (Ganesan et al., 2011). A gain-of-function allele of *mtd* exhibited an increased resistance to oral infection by the gram-negative bacteria *Vibrio cholerae* (Wang, Berkey, and Watnick 2012). As *Drosophila melanogaster* do not have an adaptive immune system that can produces antibodies that target foreign microbes (Aggrawal and Silverman 2007) they must rely on their two innate immune responses to survive. The two systems are the Toll pathway, which primarily responds to gram-positive bacteria, and the IMD pathway, which primarily responds to gram-negative bacteria (Ganesan et al., 2011). The IMD pathway is a broadly- conserved Nuclear factor kappa B (NF-kB) immune signaling pathway that regulates the antibacterial defense response (Hetru and Hoffmann 2009). For the IMD pathway to become activated, Diaminopimelic acid-type peptidoglycan (found in the cell wall of most gram-negative bacteria) is detected by two different peptidoglycan recognition protein (PGRPs), PGRP-LC and PGRP-LE which activate the IMD signaling cascade (Kleino and

Silverman 2014). Relish, which is the third NF-kB homolog in *Drosophila*, is activated by receptor protein recognition. The Dredd and IkB kinase (IkK) complexes are activated by a series of intermediates, that consist of ird5 and Kenny which encode the regulatory and catalytic subunits. The IkK complex phosphorylates the NF-kB homolog Relish, and Dredd then cleaves Relish, which releases a fragment that travels to the nucleus to activate the transcription of target genes, including antimicrobial peptides such as diptericins, attacins and drosocin which respond to gram-negative bacteria that infect the IMD pathway in order to eliminate the bacteria(Stöven et al. 2000; Hetru and Hoffmann 2009). The study by Wang et al. (2012), found that *mtd* can change how the IMD pathway responds to infection.

Taken together, the results of these previous studies suggest a hypothetical mechanism for bacterial infection to lead to a change in recombination rates. As *mtd* is part of the IMD pathway that responds to gram-negative bacteria, and loss of *mtd* function greatly reduces recombination rates, perhaps the IMD pathway is the mechanism that infected females use to increase their recombination rates. To test this hypothesis, we wanted to measure if the recombination rate changes caused by *P. rettgeri* infection depend on *mtd* function. Our hypothesis is that *mtd* function is needed to increase recombination rates in response to infection. To be able to test this, we first need to demonstrate that in our hands bacterial infection leads to a measurable increase in recombination rates.

Background and Significance

Drosophila melanogaster **development:**

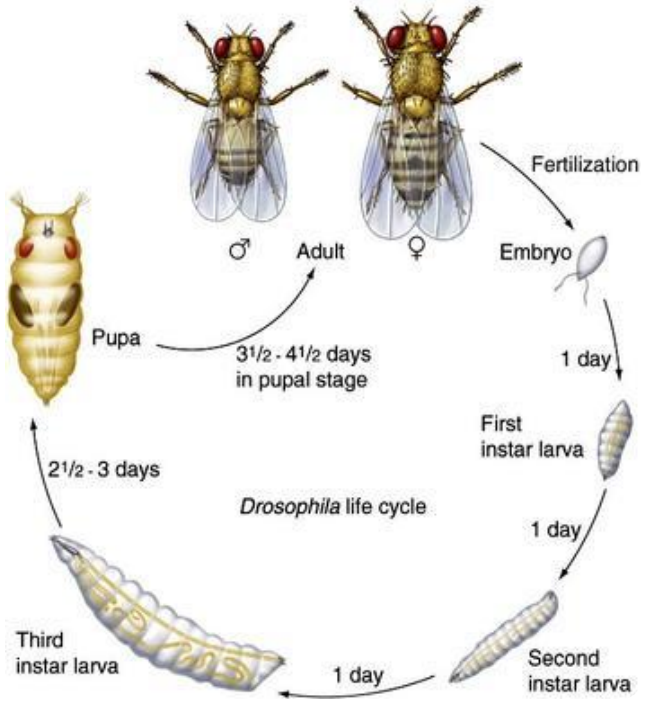


Figure 1: Life cycle of *Drosophila melanogaster*. This figure shows the lifecycle through which *Drosophila melanogaster* go from embryo to adult fly. (Creative diagnostics)

Drosophila melanogaster has been used for genetic studies for more than a century, starting with Thomas Hunt Morgan in 1910. *Drosophila melanogaster* is one of the best model organisms for studying recombination for several reasons, including the care that is needed (they require very little lab equipment to keep, they have high fecundity, they can be genetically modified, and they have a short life cycle.) Due to the rapid life cycle of *Drosophila*, genetic studies can be done quickly with large sample sizes. Fly stocks are usually kept at approximately 25°C in an incubator with 12 hour day/night illumination. At this temperature it takes approximately 10 days from fertilization to the emergence of an adult fly. There are four stages of the *Drosophila* life cycle, these include the embryo, larva, pupa and adult life stages. The embryo stage is competed within 24 hours after fertilization is completed. After the embryo stage is the larval stage which consists of three different larval molts, called the 1st, 2nd and 3rd instar larval stages. This takes about four days to be completed, with the first two stages taking one day each and the third day taking about two days. After the third instar, the larva will form a pupa to

complete metamorphosis. The pupal stage takes about four days to complete, at which point the adult fly emerges from the pupal case (Fernández-Moreno et al. 2007).

Mitosis:

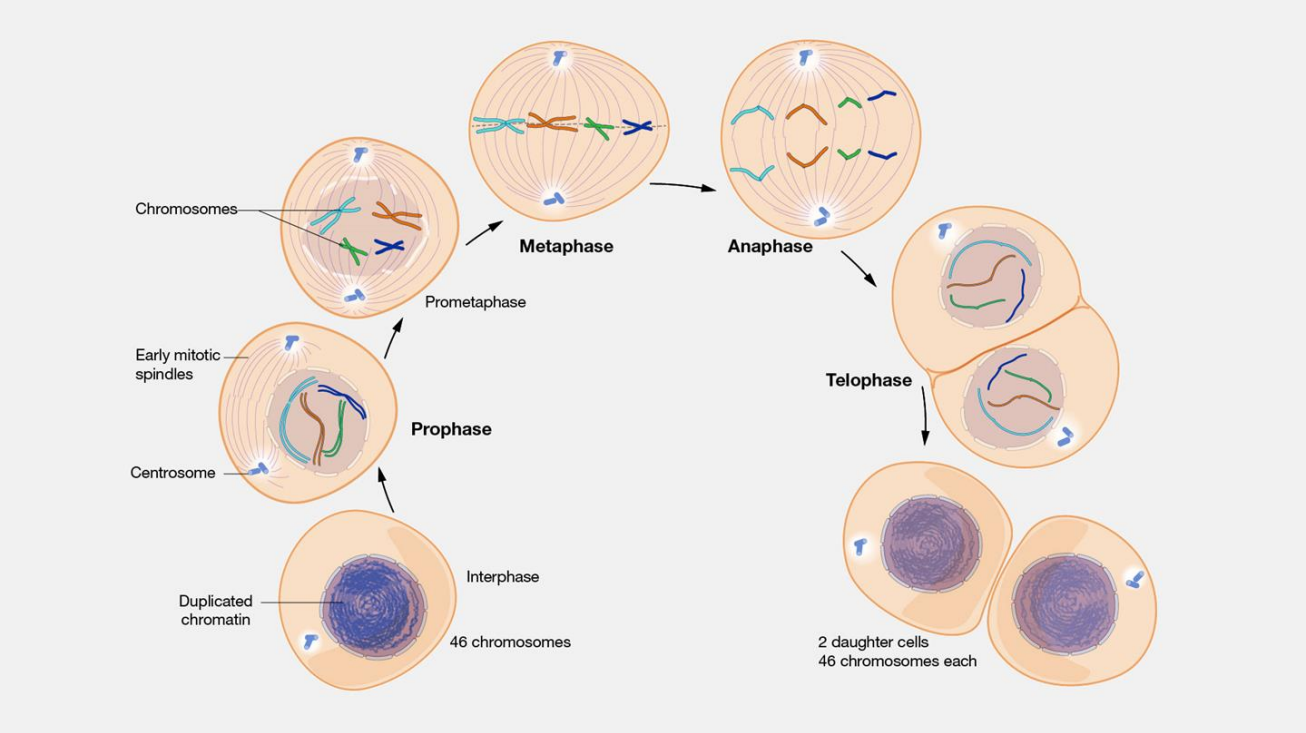


Figure 2: Schematic diagram of mitosis. The schematic demonstrates the different phases of mitosis, starting with interphase and ending with telophase/ cytokinesis. (Gilchrist, NIH Website)

Gametes (eggs and sperm) are haploid cells that are involved in sexual reproduction. They are produced by a process called meiosis which is a specialized form of cell division that results in genetically variable cells. Meiosis is evolutionarily derived from mitosis, and has many similarities to mitosis; however, while mitosis goes through one round of division and produces two genetically identical daughter cells (Figure 1) meiosis goes through two different divisions and produces four genetically unique daughter cells. Both meiosis and mitosis are preceded by interphase which begins the cell cycle.

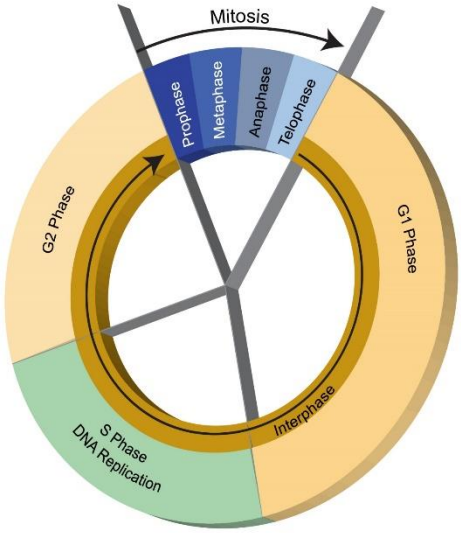


Figure 3: Schematic diagram of the cell cycle. The schematic demonstrates the different phases of the cell cycle, G1 (Gap phase 1), S (synthesis), G2 (Gap phase 2) and M (Mitosis for cell division). (Gilchrist, NIH Website)

The cell cycle is divided into four phases, these include G1 (gap phase 1), S (synthesis), G2 (gap phase 2) and M phase. G1 is the longest phase in which the cell grows and proteins that are required for cell division are synthesized (McIntosh 2016). There are checkpoints at the end of each phase that help ensure the cell has the necessary enzymes needed for DNA replication before moving onto the S phase. Once G1 has been completed the cell enters the S phase where the chromosomes are each replicated, the DNA synthesis must be completed prior to the cell undergoing mitosis. The S phase is where the cell replicates DNA in preparation for cell division. In the G2 phase the cell continues to grow and prepares for mitosis by synthesizing additional proteins and organelles that are needed to proceed to the Mitotic phase (M phase). Mitosis is the process by which a single cell divides into two identical daughter cells. This process is essential for growth, development, and repair of tissues in the body. Mitosis is a complex process that involves a series of stages, each with its unique characteristics (Rieder 2011).

There are four primary stages of mitosis: prophase, metaphase, anaphase, and telophase. In each stage, the cell undergoes various changes, such as the condensation of chromosomes, alignment of chromosomes at the equator, and the separation of the duplicated chromosomes into two distinct nuclei.

Prophase is the first stage of mitosis, during which the chromatin condenses into tightly coiled chromosomes. The nuclear envelope, which separates the nucleus from the cytoplasm, also breaks down, allowing the chromosomes to move freely within the cell. The centrosomes, which are structures that help organize the cell's microtubules, also begin to move towards opposite ends of the cell (McIntosh 2016).

In the next stage, metaphase, the chromosomes align along the equator of the cell, known as the metaphase plate. This is facilitated by the spindle fibers that attach to the centromeres, which are specialized regions on the chromosomes where the kinetochore, a molecular motor that moves the chromosomes during division, is assembled. Applying bipolar tension to the two kinetochores is how the cell detects correct biorientation of the homologs (Nicklas 1974) which ensures that each daughter cell will receive an identical copy of the genetic material.

The third stage, anaphase, begins when the enzyme Separase cleaves the Cohesin complexes holding sister chromatids, which are the two identical copies of each replicated chromosome, together. Then the spindle fibers pull the sister chromatids apart towards opposite poles of the cell (Earnshaw and Pluta 1994). This ensures that each daughter cell will receive an identical set of chromosomes.

Finally, in telophase, the chromosomes arrive at opposite poles of the cell, and the nuclear envelope reforms around each set of chromosomes, forming two new nuclei. The spindle

fibers also begin to disassemble, and the chromosomes begin to decondense, returning to their pre-mitotic state.

Cytokinesis, the process of dividing the cell's cytoplasm, typically occurs after mitosis is complete. This process results in the formation of two genetically identical daughter cells, each with its own nucleus and organelles.

Overall, mitosis is a highly regulated and precise process that ensures the accurate transmission of genetic information from one generation of cells to the next. Any errors in mitosis can lead to genetic mutations or abnormal cell growth, which can have significant consequences for the organism (Lamb et al. 2005). Because the cell cycle is ancient and conserved among all eukaryotes, studying a model organism like *Drosophila* can be informative for how it works in humans as well.

Meiosis:

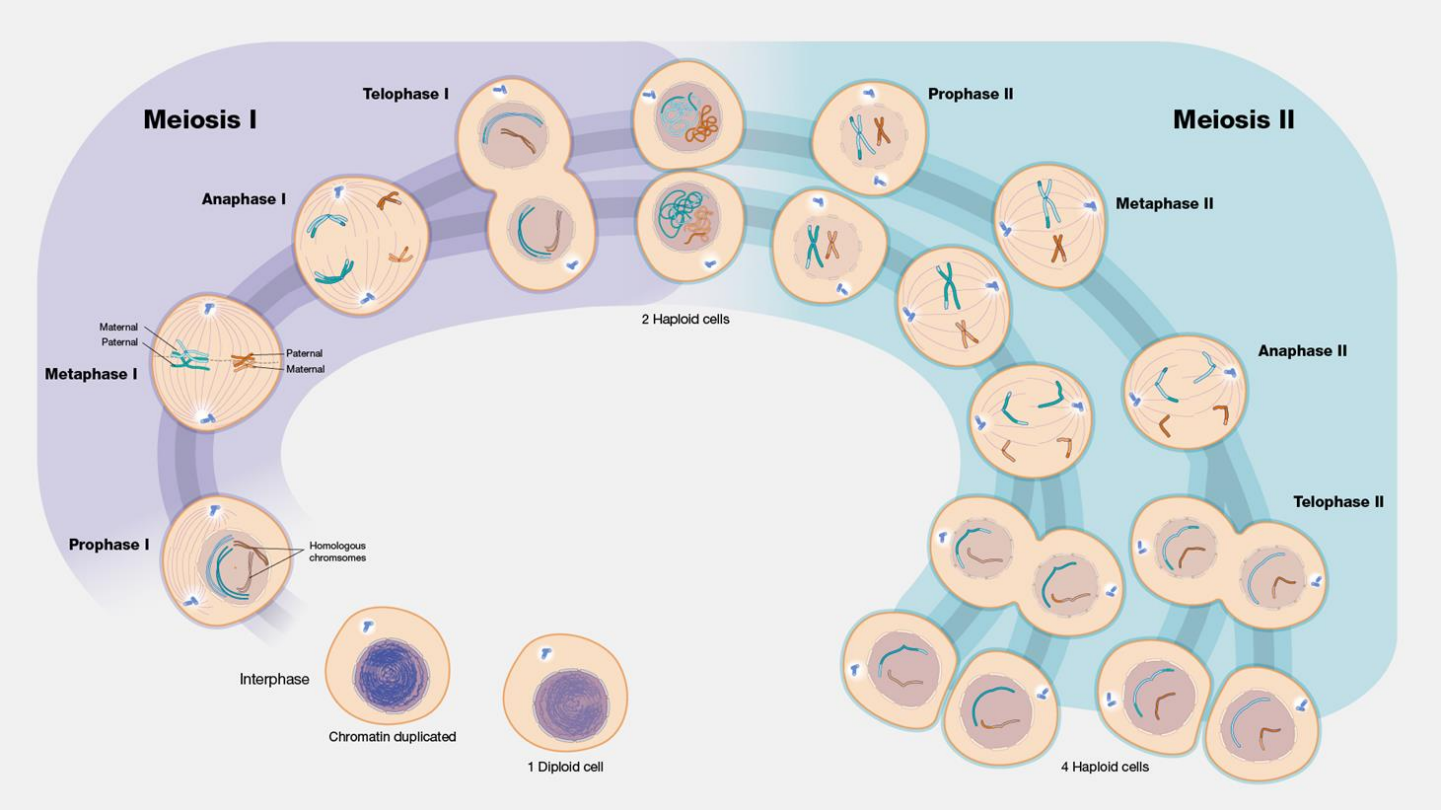


Figure 4: Schematic diagram of meiosis. Diagram of meiosis I and II showing the different phases. (Gilchrist, NIH Website)

Meiosis is the process by which cells divide to produce gametes for sexual reproduction. Unlike mitosis, which produces genetically identical daughter cells, meiosis results in the production of four non-identical daughter cells, each with half the number of chromosomes as the original cell. Meiosis evolved as a derived form of mitosis, which is why it shares many of the same steps. This reduction in chromosome number is critical for the progeny to receive the correct number of chromosomes after fertilization (Lenormand et al. 2016).

One key difference between mitosis and meiosis is that in mitosis the chromosomes are copied once and the cell divides once, whereas in meiosis the chromosomes are copied once, followed by two rounds of cell division. This is why meiosis occurs in two stages, meiosis I and meiosis II, each consisting of Prophase, Metaphase, Anaphase, and Telophase (Figure 2). The two stages of meiosis are separated by a period of cellular rest called interkinesis.

Oogenesis:

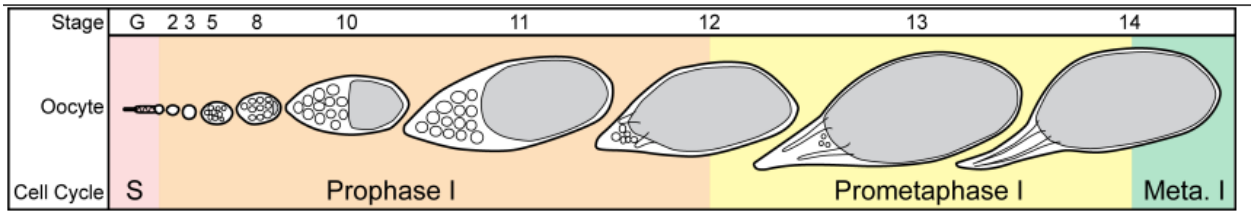


Figure 3: Ovariole Structure and Stages of Oogenesis. The ovariole is a string of developing cysts, divided into 14 stages (not all shown) based on oocyte morphology (gray), polytene nurse cells (white circles) and dorsal appendages (black lines). Stages correlate to progress through the meiotic cell cycle (Cell Cycle; colors), so Stage 12/13 is the transition from Prophase I to Prometaphase I, while Stage 14 oocytes are at Metaphase I arrest. This allows the inference of cell cycle stage using oocyte morphology. Figure adapted from Sullivan et al 2000.

Meiosis and oogenesis, the process of egg cell (oocyte) development in female organisms, are tightly linked, with the morphology of the egg correlating to progression through

the cell cycle. Oogenesis occurs in specialized organs called ovaries, which occupy around half the volume of a female fly's abdomen. The two ovaries in a *Drosophila* female each contain approximately 16 to 20 ovarioles, which are assembly-line factories for producing eggs. (McLaughlin and Bratu 2015). There are 14 stages of oogenesis (Figure 3) in *Drosophila*, which were defined by distinct changes in the morphology of the developing oocyte (King 1970; Sullivan, Ashburner, and Hawley 2000). The germline stem cells are in the germarium at the anterior tip of the ovariole, and developing egg cysts move posteriorly as they mature. Recombination is finished by stage 2a (Hughes et al. 2018), very early in oogenesis, and then prophase ends at germinal vesicle breakdown at stage 12, reaching metaphase in mature stage 14 oocytes. The oocyte then arrests to await fertilization and oviposition.

Meiosis I begins with prophase I, which is the most complex and extended phase of meiosis. During prophase I, homologous chromosomes pair up and undergo crossing over, a process in which segments of genetic material are exchanged between chromatids. The paired chromosomes are held together by a protein complex called the synaptonemal complex, which is required for recombination to occur in *Drosophila* (Page and Hawley 2004). In addition to locking homologous chromosomes together so they can co-segregate, this process shuffles the genetic material on the parent's two chromosomes, creating new combinations of genetic information, resulting in increased genetic diversity among the offspring.

Prometaphase I occurs between prophase I and metaphase I. This stage begins when the nuclear envelope breaks down, which allows the microtubules to come in contact with the chromosomes. The spindle fibers begin to attach to the kinetochores, protein structures on the centromeres of chromosomes (Wignall and Villeneuve 2009; Muscat et al. 2015). Once the kinetochores are attached, the chromosomes move into a single mass at the metaphase plate, a

process called congression. The spindle fibers exert force on the chromosomes through the kinetochores, which pulls them towards the center of the cell. Once congression is complete, the chromosomes are properly co-oriented and ready to segregate, ensuring each daughter cell receives the correct genetic material. At this point, the cell has reached metaphase I arrest, with the homologous chromosome pairs aligned at the metaphase plate. This alignment is random, meaning that each pair of maternal and paternal chromosomes can be oriented in any combination. This independent assortment of chromosomes further increases genetic diversity.

Like in mitosis, Anaphase I occurs when the Cohesin holding sister chromatids together is cut, leading to the separation of the homologous chromosomes to opposite poles of the cell. One key difference from mitosis is that while in mitosis the sister chromatids segregate from each other, in meiosis I the homologous chromosomes segregate. To keep the sister chromatids attached at the centromere, a small amount of Cohesin is protected by the protein Shugoshin (Watanabe and Kitajima 2005), which holds sister centromeres together until they separate in meiosis II.

Telophase I involves the formation of two new nuclei around the separated chromosomes. Cytokinesis then occurs, resulting in the formation of two new haploid daughter cells, each with half the number of chromosomes as the original cell, but each chromosome already having two sister chromatids.

Meiosis II is similar to mitosis in that the sister chromatids are separated in the same way as in anaphase of mitosis. The main difference is that there is no replication of DNA between meiosis I and meiosis II. Therefore, the daughter cells of meiosis II each receives a single copy of each chromosome, rather than the two copies present in the original cell.

Overall, meiosis is a highly regulated process that ensures the production of genetically diverse gametes. The genetic diversity introduced by meiosis is essential for the survival and evolution of species, as it allows for the production offspring with new combinations of traits that may be better suited to their environment. Any errors in meiosis can lead to genetic abnormalities or disorders, which can have significant consequences for the offspring.

Aneuploidy occurs when there is an abnormal number of chromosomes in a cell, meaning that there are more or fewer chromosomes present than normal. This can occur through nondisjunction which is when homologous chromosomes or sister chromatids fail to properly separate at some point during cell division. Having an abnormal number of chromosomes is the most common cause of human birth defects and includes conditions such as Down syndrome and Turner syndrome (Lamb et al. 2005).

The result of meiosis and recombination is the formation of genetically diverse haploid cells. These cells will eventually mature into gametes (sperm or egg cells), which are combined during fertilization to form a new diploid offspring that carries a unique combination of genetic material from both parents (Hughes et al. 2018).

Recombination:

Recombination occurs through the rearrangement of DNA sequences in which two homologous chromosomes align and then a double-strand break is made in one of the molecules. The cut is resected to produce single-stranded DNA on each side of the cut. These single strands then undergo strand invasion, pairing with the double-stranded homolog and forming a heteroduplex DNA structure known as a Holliday junction. How this structure is resolved can lead to either a crossover or non-crossover outcome (Song et al. 2022). More double strand

breaks are formed than crossovers, with most double-strand breaks being resolved to non-crossover gene conversion events. If a crossover does occur, it forms a physical connection between the homologs that produces the tension between homologs that allows accurate segregation to happen (Smith and Nambiar 2020).

Recombination is an important process in that it contributes to genetic variability. The Red Queen hypothesis proposes that organisms must constantly adapt and evolve to maintain their relative fitness within a changing environment (Clay and Kover 1996; Mostowy and Engelstädter 2012). One prediction of this hypothesis is that when a host is infected by pathogens, if it can diversify its offspring, it increases the chances that some offspring will be able to resist the pathogen and survive. A study by Singh et al. (2015) showed that recombination rates were increased in the offspring of female flies that were infected with a gram-negative bacterium *Providencia rettgeri*, when compared to wounding-only controls. This was the first study to show that pathogens could increase recombination rates and lead to more diversified offspring in animals.

Mustard gene:

The Gilliland lab studies the processes of chromosome movement through meiotic prometaphase I and how they congress to the metaphase plate. To identify new genes required for the process, the lab conducted a genetic screen to identify genes that cause defects in congression when knocked down by RNA interference (RNAi). One of the genes that this screen identified was *mustard* (*mtd*), which had 100% congression failure in the initial screen. The *mtd* gene was originally an uncharacterized lethal gene known as a late puff gene, l(3)82Fd, which caused a failure to emerge from the pupal case when mutated (Stowers et al., 1999). A later study recovered this gene in a genetic screen for mutations that can alter host susceptibility to infection

by *Vibrio cholerae*, a gram-negative bacterium that causes the disease diarrheal cholera and can also infect the *Drosophila* gut (Wang, Berkey, and Watnick 2012). The screen was done due to IMD pathway mutants possibly having an increased tolerance to *V. cholerae* when orally infected (Berkey, Blow, and Watnick 2009). Wang and coworkers were using *Drosophila* as a model for cholera, feeding bacteria to flies and assaying different genotypes; they found that the intestinal adherens junctions of *mtd* and other IMD pathway mutants were better protected from infection when compared to controls (Wang et al. 2013). Because they found this gene interacted with *Relish*, a part of the IMD innate immunity pathway, Wang et al. named this gene *mustard. Mtd* and the IMD pathway have also been shown to regulate intestinal stem cell division separately from when they are infected by intestinal bacteria (Wang et al. 2013). The Human orthologs of *mtd*, Oxr1 and NCOA7, have also been shown to mediate oxidative stress response and protect against reactive oxygen species (Oliver et al. 2011).

IMD Pathway in *Drosophila melanogaster*:

Because *Drosophila melanogaster* do not have an adaptive immune system to make antibodies that target foreign microbes, when flies are infected by microbial pathogens they must rely on their innate immune responses that produces a nonspecific response to invading pathogens by synthesizing antimicrobial peptides (Aggrawal and Silverman 2007). *Drosophila melanogaster* has two different innate immune signaling pathways, the Toll pathway, and the immune deficiency pathway (IMD). The Toll pathway is mainly activated by gram-positive bacterium, whereas the IMD pathway is mainly activated by gram-negative bacteria (Wang, Berkey, and Watnick 2012). The nuclear transcription factor NF-kB is an important part of the host defense in *Drosophila* as it controls the expression of the genes that encode immune-responsive peptides, diptericin, attacin and drosocin (Hetru and Hoffman, 2009). Nuclear translocation of NF-kB homologs is regulated by both pathways, and the regulation of NF-kB in turn activates the transcription of many other genes (Wang, Berkey, and Watnick 2012). In the *Drosophila melanogaster* genome, there are three NF-kB homologs, two that respond to

signaling through the Toll pathway and one, *Relish,* that responds to signaling through the IMD pathway (Wang, Berkey, and Watnick 2012). *Relish* is activated by receptor protein recognition, which causes it to respond to signaling through the IMD pathway (Figure 4). The Dredd and IkK complexes are activated by a series of intermediates, which consist of *ird5* and *Kenny* which encode the regulatory and catalytic subunits. The IkK complex phosphorylates the NF-kB homolog Relish, and Dredd then cleaves Relish, which releases a fragment (Rel-68) that travels to the nucleus to activate target genes (Wang et al., 2012). In *Drosophila* it is thought that the innate immune response to commensal and pathogenic bacteria in the gut is activated by IMD pathway signaling (Ryu et al. 2006; Buchon, Broderick, and Lemaitre 2013).

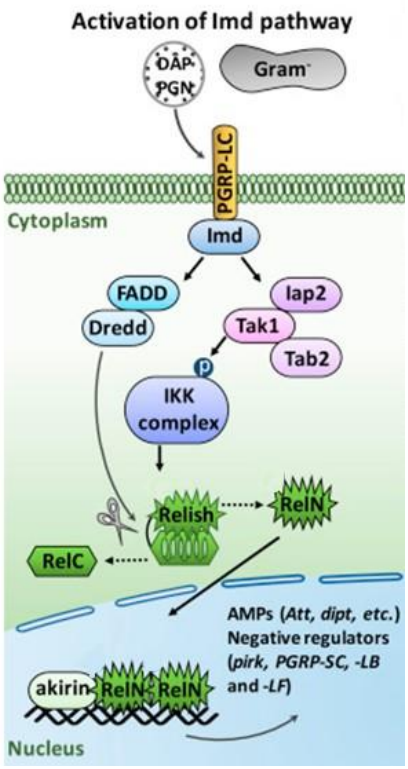


Figure 5: Diagram of IMD Pathway in *Drosophila melanogaster.* For the activation of the IMD pathway, transcription factors Relish and NF-kB are required. Adaptor protein IMD is recruited by PGN and then phosphorylated and activates downstream signaling cascade. The IkK complex phosphorylates the NF-kB homolog Relish, and Dredd then cleaves Relish, which releases a fragment that travels to the nucleus to activate target genes (Adapted from (Salminen and Rämet 2016).

Previously, *mtd* was not known to be involved in meiosis and was only known for its role in the IMD pathway. One explanation for the high rates of congression failure when *mtd* is compromised could be that it is involved in the regulation of recombination. Other mutants that block recombination, such as *c(3)G* and *meiW68*, have similar patterns of congression failure (Gilliland et al. 2009), which suggests the possibility the loss of *mtd* also causes recombination rates to be reduced. Because Singh et al (2015) found that infection by gram-negative bacteria increased recombination rates, it is possible that *mtd* is part of the mechanism that increases recombination rates in response to infection.

To determine if *mtd* is part of the mechanism that increases rates in response to infection, we examined whether the loss of *mtd* in the germline leads to changes in recombination rates. To do this we used two different drivers to trigger RNAi, which had the Gal4 transcriptional activator under control of the *nanos* and *matalpha* promoters. *Nanos* turns on at the start of oogenesis before recombination is completed while *Mat-alpha*, a female meiosis-specific alpha tubulin subunit, turns on shortly after recombination has been completed (Matthews, Miller, and Kaufman 1989). We predict that there will be a reduction in recombination rates when *mtd-RNAi* is driven with *nos::Gal4*, but not with *matα::Gal4*. Second, we also predict that *mtd* is required to modulate recombination rates in response to bacterial infection. To test this hypothesis, we first need to demonstrate that bacterial infection increases recombination rates in control flies. These flies will be infected with *P. rettgeri* bacteria by septic pinprick, and their recombination rates measured. If this reduces recombination rates, we can then perform the same experiments in flies where *mtd* has been knocked down. We predict that without *mtd* there will be no increase in recombination rates.

Materials and Methods

Fly Stocks:

The fly stocks that were used during this project were maintained by Dr. William Gilliland and me. The flies were reared in bottles and vials that contained Nutri-fly Bloomington formula fly food (www.geneseesci.com) and transferred to fresh food every 21 days. The vials and bottles were kept at 25℃ in an incubator that was set to a 12-hour light and dark cycle.

List of Fly Stocks:

For these experiments, we used the following stocks that were maintained by the Gilliland lab. The multiply marked *y cv v f* stock was used to measure crossing over on the *X*, and was crossed to standard wildtype laboratory strain Oregon-R. The RNAi construct targeting *mtd* came from Bloomington Drosophila Stock Center (BDSC) stock #36638, *y sc v sev; P{y$^+$ v$^+$=TRIP.GL00598}attP40*. This was crossed to the *y cv v f* stock to create the genotype *y w; P{y$^+$ v$^+$=TRIP.GL00598}attP40.* The *nos::Gal4* construct came from the stock *y w / y$^+$Y; nos-Gal4:VP16; pol.* The *matα::Gal4*driver came from BDSC stock #7063, *w; P{w^{+mC}=matα4-GAL4-VP16}V37*.

Fly crosses and Recombination rate assays:

y cv v f / y$^+$ cv$^+$ v$^+$ f$^+$ recombination rate assay:

Homozygous mutant females (*y cv v f / y cv v f*) were crossed to Oregon-R (*y$^+$ cv$^+$ v$^+$ f$^+$*/ Y) male flies and allowed to lay eggs in bottles. Homozygous mutant females were *yellow* bodied (*y*), *cross veinless* winged (*cv*), *vermillion* eyed (*v*) and *forked* bristled (*f*). Oregon- R males were the

wildtype flies; their phenotypes for all of these markers were wildtype. On day 6, adult flies were dumped from the bottles. Collection of virgin female flies started on day 10 and proceeded until approximately day 15, or until enough females had been collected. Virgin females were heterozygous (*y cv v f/y*$^+$ *cv*$^+$ *v*$^+$ *f*$^+$*)* and males were homozygous mutants (*y cv v f* /Y). Females were placed into previously yeasted vials with 2-3 males per vial. Adults were dumped from vials on approximately day 6. Counting of progeny started on day 10 and continued until day 18. Following completion of counting offspring, recombination calculations were conducted.

We calculated the recombination rate frequency using the counts of progeny carrying the four different marker alleles. A recombination can be detected when the linkage of adjacent markers changes; for example, a *y cv*$^+$ or *y*$^+$ *cv* progeny must have undergone a recombination in the *y-cv* interval. %RF_1 refers to the number of recombinant progeny in the *y-cv* interval, %RF_2 to those in the *cv-v* interval and %RF_3 to those in the *v-f* interval. Double recombinant progeny added one recombinant to each of the two intervals involved. The combined totals of recombinant progeny for each interval was then divided by the total number of progeny, including non-recombinant, single recombinant and double recombinant offspring (N=total).

$$y \underset{1}{—} cv \underset{2}{—} v \underset{3}{—} f$$

$$\% RF_1 = 1 \text{ Only} + (1 \& 2) + (1 \& 3)/ N \times 100\%$$

$$\% RF_2 = 2 \text{ Only} + (1 \& 2) + (2 \& 3)/ N \times 100\%$$

$$\% RF_3 = 3 \text{ Only} + (1 \& 3) + (2 \& 3)/ N \times 100\%$$

One percent of recombinant progeny in this assay is referred to as “one map unit”, and is also called a centiMorgan, or cM.

cv f/cv^{+} *f*$^{+}$ recombination rate assay:

The y^{+} and v^{+} markers carried by the RNAi construct inhibited the ability to use all the markers of *y cv v f*, because a y^{+} or v^{+} progeny could have received the wildtype allele from either the *X* chromosome or from the RNAi construct. Therefore, we used the same methods as described in the previous section for the *y cv v f/y*$^{+}$ *cv*$^{+}$ *v*$^{+}$ *f*$^{+}$ recombination rate assay, but only considered the phenotypes for *cv* and *f*. This limits recombination rate data to a single interval.

mtd-RNAi crosses:

The induction of RNA interference requires a short palindromic hairpin sequence (the "RNAi construct") that matches the gene of interest. This construct will only be transcribed when the Gal4 transcriptional activator protein is expressed in the same tissue. We used two different drivers, *nos::Gal4,* which expresses Gal4 under control of the master germline transcription factor *nanos,* inducing RNAi at the initiation of oogenesis, and *matα::Gal4*, which expresses somewhat later in oogenesis. By crossing a stock carrying the RNAi construct to a stock that expresses Gal4 protein under control of a tissue-specific promoter, the gene of interest can be knocked down only in the desired tissue.

Providencia rettgeri culturing:

LB Broth preparation and plate preparation

Luria broth (LB) was prepared by adding 20g of LB powder to 500mL of ddH_2O. Bottle was shaken until powder was fully dissolved in water. The solution was then placed in autoclave for sterilization. After the sterilization process was complete the bottle was of LB broth was left to

cool slightly before pouring onto plates. To ensure sterility of the solution bottle was opened in the vicinity of an open flame. Liquid was poured onto the plates, enough to fully cover the bottom of the plates. Plates were left over night at room temperature. For long term storage the plates were placed in 4°C fridge.

Growth of *Providencia rettgeri*

Our culture of *P. rettgeri*^{Dmel} was a generous gift from Dr. Brian Lazzaro of Cornell University. Bacteria were streaked onto LB agar plates from a -80°C glycerol stock and grown in a 37°C incubator overnight. Plates were then kept at 4°C for long term storage.

Inoculation of female *Drosophila melanogaster* with *Providencia rettgeri*:

Preparation of bacterial culture

Bacterial culturing followed published protocols (Khalil et al. 2015). Briefly, a bacterial culture of *P. rettgeri* was prepared overnight by inoculating 2ml of sterile liquid LB with one colony of *P. rettgeri* picked from the growth plate. The culture was then grown overnight at 37°C with gentle shaking (150rpm). After overnight incubation 600ul of the culture was transferred to an Eppendorf tube and centrifuged for three minutes at 5000g. The supernatant was then removed and 1000ul PBS was added and vortexed to resuspend bacteria. Bacteria was then diluted by adding PBS to a density between A_{600} OD (0.1- 1.0).

Needle preparation

To prepare the needles for inoculation p200 pipette tips and 0.15 mm insect pins were used. The p200 pipette tips were lightly melted over a Bunsen burner and the blunt end of the insect pin

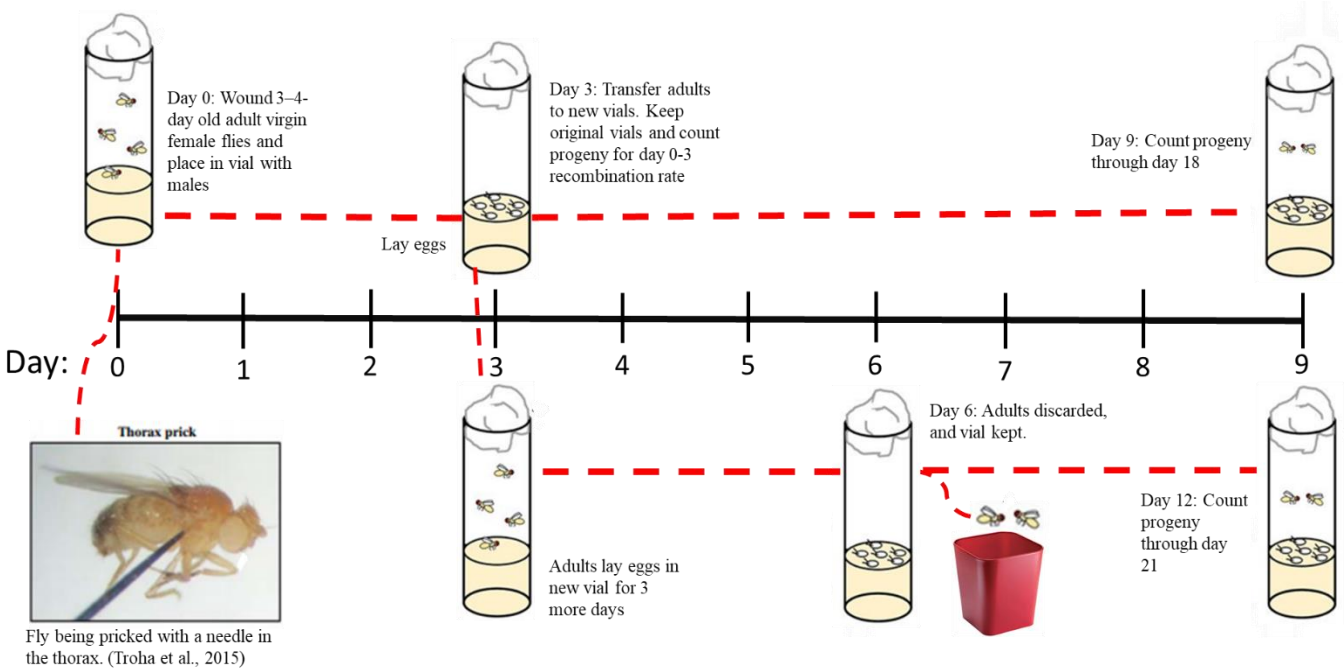
was inserted 5mm into the melted plastic. The pins were held for a few seconds until the plastic was cooled enough for the pins to set.

Preparation of flies and vials

Virgin female drosophila were collected earlier in the week on Mondays and Tuesdays, to ensure that they were aged between 3 and 4 days. They were aged between 3 to 4 days to ensure that recombination would be completed prior to being inoculated. Vials were prepared with 1-3 females per vial and 2-3 males per vial.

Inoculation of flies

Females were inoculated using the previously prepared needles. The needles were dipped into either the diluted bacteria or into PBS. Females were pricked in the sternopleura with the tip of the needle, to an approximate depth of 0.2mm. After female flies were inoculated, they were placed in vials with males, leaving the vial on its side until females had recovered.



Fly being pricked with a needle in the thorax. (Troha et al., 2015)

Figure 6: Experimental plan demonstrating the timeline of wounding and infecting female drosophila. On day zero flies are either wounded with sterile PBS or infected with *P. rettgeri* and are placed in vials with 2-3 male flies. They spend 3 days in one vial before being moved to a second vial on day 3. On day 6 they are discarded from the vial. Counting begins on day 9 for the first vial and day 12 for the second vial.

Recombination rate assay with *Providencia rettgeri* bacterial infection:

Bottles of virgin *y cv v f* females crossed to Oregon- R males are set-up ten days prior to collection. On days 10 and 11 virgin females are collected and held in vials until they have been aged between 3-4 days old. The 3–4-day old virgin females were then inoculated with either *P. rettgeri* for the experimental group or with PBS for the control group. After flies were inoculated, they were given the opportunity to recover for a few minutes. Approximately 2-3 *y cv v f* males were added to the vials. The vials were then checked after 72 hours and vials with surviving females were then moved to fresh vials for another 72 hours. Because oogenesis takes three days, the eggs laid during the first 72 hours will have completed recombination prior to infection. After 72 hours, the eggs laid would have completed recombination after infection. Progeny was then counted starting on day 10 post placement in the pre-infection vials, the counting of progeny also started on day 10 for post-infection vials. Counting of progeny was continued up to day 18 for both pre and post infection numbers (Figure 5).

Results

To measure the normal recombination rates without bacterial infection, we crossed *y cv v f*/ + + + + females and counted the progeny for recombination in the three intervals. On the *y-cv* interval the map distance was 7.4 cM, *cv-v* was 19.7 cM and the *v-f* was 24.4 cM (Table 1). To

get the crossover rate for the *cv-f* interval, the *cv-v* and *v-f* rates were combined, resulting in a map distance of 43.6 cM. For the *mtd*-RNAi construct, only the *cv-f* interval was able to be to scored, due to the RNAi construct also being marked with y^+ and v^+. The map distance for *cv-f* in *mtd*-RNAi females was 1.8 cM. This represented a reduction of the recombination rate by 96% when *mtd* is knocked down by RNAi, confirming our prediction that *mustard* causes congression failure and nondisjunction by reducing crossing over.

Recombination (*y cv v f*/ + + + +)	***mtd*-RNAi (no infection)**
$\boldsymbol{y-cv}: \frac{185}{2499} \cdot 100 = 7.40$	
$\boldsymbol{cv-v}: \frac{491}{2499} \cdot 100 = 19.65$	$\boldsymbol{cv-f}: \frac{58}{3321} \cdot 100 = 1.75$
$\boldsymbol{v-f}: \frac{610}{2499} \cdot 100 = 24.4$	

Table 1: Map distances for Normal Recombination: *y-cv* = 7.4 cM, *cv-v* = 19.7 cM, *v-f* = 24.4 cM . Map distance for *mtd*-RNAi: *cv-f* =1.8 cM

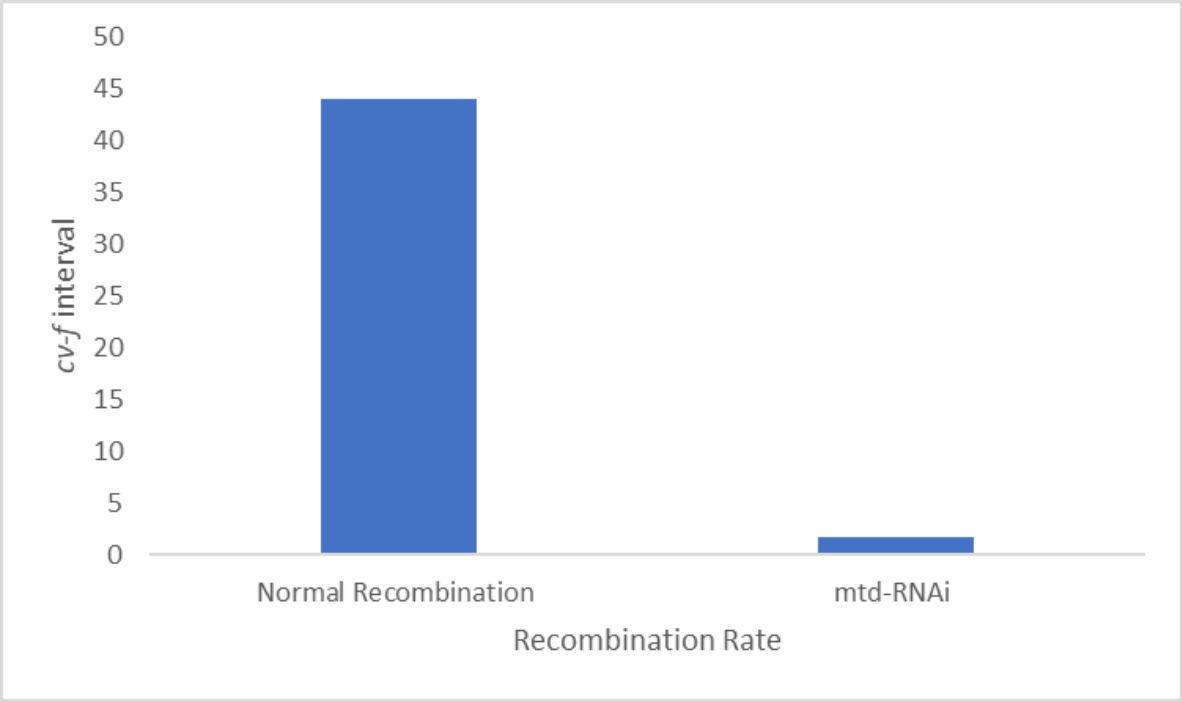


Figure 7: Crossover rates for females with normal *mtd* and females undergoing *mtd* -RNAi (without infection). The *y* and *v* markers cannot be scored in the mtd-RNAi experiment since the construct is marked with y^+ and v^+. The intervals *cv-v* and *v-f* were combined in the normal treatment, resulting in a *y-cv* map distance of 43.6 cM in the normal females, compared to 1.8 cM in *mtd*-RNAi females. This means that RNAi of *mtd* reduced recombination rates by 96%.

Because recombination occurs early in meiosis, we were able to drive RNAi with *matα::Gal4* that does not induce RNAi until oocyte stage 2a, which is after recombination has completed. Therefore, if *mtd* is causing nondisjunction (NDJ) due to a lack of recombination, then we should not see a defect when RNAi is driven by *matα::Gal4*. This is indeed the case; when the *mtd*-RNAi was driven by *nos::Gal4*, we observed 18.6% X and 9.6% 4 NDJ. But when the same RNAi construct was driven by *matα::Gal4*, we only observed 0.2% X and 0.1% 4 NDJ, similar to normal levels.

The results from these recombination assays showed that *mtd* is required for normal recombination to occur. To see if *mtd* is also required for bacterial infection to alter recombination rates, we next needed to determine if these rates were changed in normal females that were infected by bacteria. Therefore, we inoculated *y cv v f*/ + + + + flies with *P. rettgeri* and repeated the previous recombination assay. The control female flies were wounded with needles dipped in sterile PBS while the experimental female flies were wounded with needles dipped in a solution of *P. rettgeri*. Both control and experimental flies were allowed to lay eggs for three days, which represented eggs that finished recombination prior to wounding. Females were then transferred to fresh vials and allowed to lay eggs for three more days. Because oogenesis requires 72 hours, and recombination is completed very early in the process, the eggs in the first vial should have undergone recombination prior to infection, while the eggs in the second vials should reflect the post-infection recombination rates. In these experiments, the majority of females in the control group survived the wounding with PBS and were able to produce progeny. In the experimental group, we expected around half of the females that were infected with bacteria to die, which is typically what occurs in this procedure (Khalil et al. 2015). On average, control females produced around 600 progeny that completed recombination pre-wounding and 459 progeny that completed recombination after wounding, while experimental females produced on average around 358 progeny that completed recombination pre-infection and around 234 progeny that completed recombination post-infection. In both the control and experimental groups, all three intervals were scored. We found that the wounding-only females had map distances of *y-cv* 7.3 cM, *cv-v* 18.7 cM and *v-f* 21.4 cM pre-wounding and post-wounding was *y-cv* 9.11 cM, *cv-v* 20.8 and *v-f* 19.7 cM while the infected females had map distances of *y-cv* 7.80 cM, *cv-v* 19 cM and *v-f* 20.9 cM pre-infection and post-infection was *y-cv*

7.04 cM, *cv-v* 19.5 cM and *v-f* 19.5 cM (Table 2). This indicated that bacterial infection did not result in an increase in the recombination rate in any interval before and after infection, and actually caused a slight decrease in recombination in each interval. Because of this failure to reproduce the published result from Singh et al., we were unable to go on to test if bacterial infection might interact with the knockdown of *mtd*.

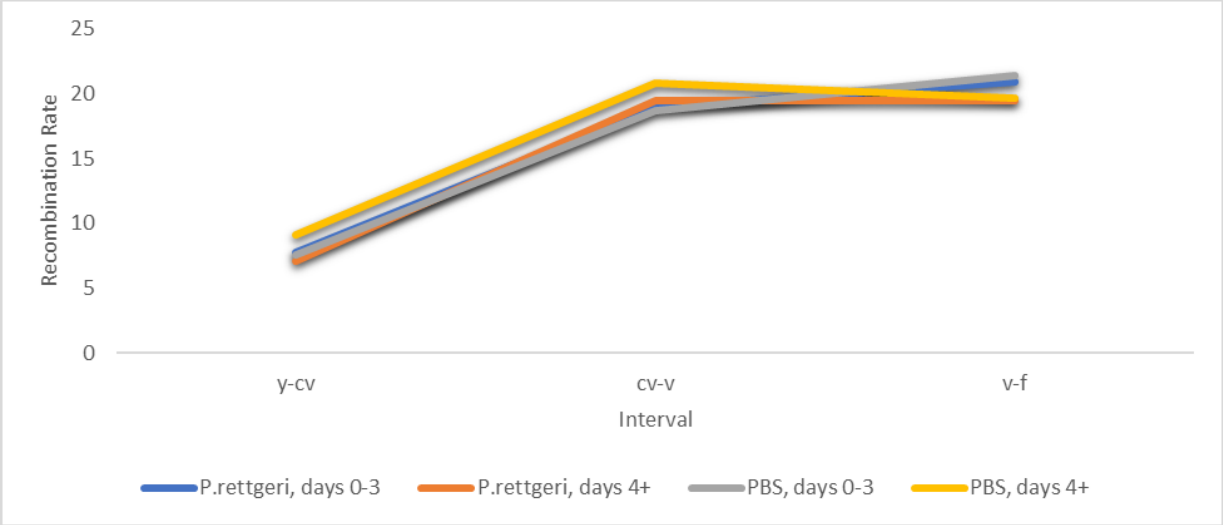


Figure 8: Recombination rates for PBS (Control) and *P. rettgeri* (Experimental) for the first 3 days and 4+ days. The recombination rates of the intervals are shown during each treatment group.

Treatments	***y-cv***	***cv-v***	***v-f***
P. rettgeri, 0-3 days	$\frac{168}{2152} \cdot 100 = 7.80$	$\frac{408}{2152} \cdot 100 = 19$	$\frac{451}{2152} \cdot 100 = 20.9$
P. rettgeri, 4+ days	$\frac{99}{1407} \cdot 100 = 7.04$	$\frac{275}{1407} \cdot 100 = 19.5$	$\frac{275}{1407} \cdot 100 = 19.5$
PBS, 0-3 days	$\frac{271}{3597} \cdot 100 = 7.53$	$\frac{675}{3597} \cdot 100 = 18.7$	$\frac{768}{3597} \cdot 100 = 21.4$
PBS, 4+ days	$\frac{251}{2753} \cdot 100 = 9.11$	$\frac{574}{2753} \cdot 100 = 20.8$	$\frac{543}{2753} \cdot 100 = 19.7$

Table 2: Results of *P. rettgeri* pre-infection and post-infection and PBS pre-wounding and post-wounding on the three intervals *y-cv*, *cv-v* and *v-f*.

Discussion

We were successfully able to measure recombination in *mtd-RNAi* females. While the normal *cv-f* interval was measured to be 43.6 cM, the map distance was reduced to 1.75 cM when *mtd-RNAi* was driven by *nos-Gal4.* However, when the driver used was *mata-Gal4,* which would not ablate *mtd* until after recombination had been completed, then we did not see an increase in meiotic nondisjunction. These experiments confirmed our hypothesis that the congression failure and nondisjunction seen in *mtd-RNAi* females was caused by a reduction of recombination rates.

However, the experiments to test the interaction between bacterial infection and *mtd* could not be conducted. When we infected the control *y cv v f / + + + +* females, we did not see an increase in the recombination rates as we had been expecting; in fact, the rates were slightly decreased. This result was unfortunate but not entirely unexpected; it was reported (Singh et al. 2015; Hunter et al. 2016) that while some wild strains of *Drosophila* did increase their recombination rates in response to infection, others did not. This suggests that there may be segregating genetic variation in natural populations that leads to this increase, and the single *y cv v f/ + + + +* genotype that we were testing may just not have been a responsive genotype. In the the trials with the wounding-only *y cv v f/ + + + +* flies, we did observe that the recombination rates were the same, or even slightly increased in the wounded females [figure 5]. There are multiple possible explanations for why this experiment was not successful.

One possibility for why we did not see an effect on recombination rates due to infection is that it is due to a chromosome specific effect. In the Singh et al (2015) study, they were looking at marker genes located on the second chromosome, whereas the genes we used are

located on the *X* chromosome. It is known that some mutants that affect recombination can cause nondisjunction in a chromosome-specific fashion (Sekelsky et al. 1999) , therefore bacterial infection may simply not increase recombination rates on the *X* chromosome. However, because the transgenes for the GAL4 driver and the RNAi construct were located on the autosomes, reconfiguring our experiment to measure recombination rates on those chromosomes was not feasible. A second possibility is that some meiotic mutants are known to change recombination rates in a polar fashion, reducing recombination rates near centromeres while increasing it near telomeres (Page et al. 2000). Because we were only measuring recombination in a single interval, this type of alteration may not have been detectible in our experiment. A third explanation for not observing an increase in recombination rates is that there could be differences in alleles that cause some strains to be affected by bacterial infection while others are not; for example, Singh et al (2015) reported that strain RAL40 did not increase its recombination rate, while strain RAL73 did. Furthermore, Hunter et al. (2016) found that there was a significant effect of Wolbachia infection on recombination rates in the *y v* interval but not in the *e ro* interval. Therefore, the same strain-specific response could be occurring in the genotypes that we used, and the *y cv v f* genotype may not be one that is affected by *P. rettgeri* bacterial infection.

A final possible explanation of our results is that the positive results in the published studies were not actually caused by recombination rate changes, but were instead due to viability differences in the recombinant progeny. Viability and recombination are confounded, because only surviving adult flies can be counted in these assays. Therefore, an increase in the number of a particular recombinant genotype being produced would be indistinguishable from an increase in the number of that genotype surviving the bacterial infection. If two alleles present on the two recombining chromosomes would result in a higher survival rate when transmitted together, this

would lead to selection that increases the number of that genotype among the surviving progeny, even though they were produced at the same rate. This outcome would be indistinguishable from an increase in the rate of recombination, which would lead to an increase in the number of that genotype being produced, even though those genotypes would survive at the same rate. Therefore, it is possible that the particular strains that Singh *et al* reported to have increased recombination rates in response to infection might have not actually increased recombination in response to infection at all, but instead certain classes of recombinant progeny might have had higher survival rates, because the recombining strains would have carried alleles on the two chromosomes that would result in recombinants surviving more frequently when transmitted together. These alleles, linked in *trans*, would only both be transferred to progeny when a recombination occurred within the interval being measured, which would lead to an increase in the survival of recombinant progeny when compared to nonrecombinant progeny. It is possible that in our *y cv v f* / + + + + genotype, there were no favorable alleles for recombination to bring together, which could explain why we did not observe increased rates of crossing over in response to infection. This hypothesis could theoretically be tested by repeating the experiment using a chromosome that has already undergone this favorable recombination, and would therefore start out having the two advantageous alleles already linked in *cis*. If this was the case, this hypothesis would predict that recombination would now break up the favorable association of those alleles, and this would result in a reduction in the survival rates of recombinant progeny compared to nonrecombinants in response to bacterial infection. However, if the strain differences are not due to viability affects, then infection should still be found to increase the recombination rate even though the markers are starting out in *cis.*

While it might have been possible to test a number of different strains and identify one that was responsive, we chose not to pursue this experiment because inoculating the flies was an arduous and labor intensive task, and we had no guarantee that the procedure would be successful. Due to the size of the flies, a small 0.15 mm needle was needed to infect them, which made it challenging to infect the flies because it was quite easy to pierce too far into the sternopleura and cause the fly more harm than they could successfully recover from. After wounding/infection occurred, half of the infected females are expected to die. However, in our hands fewer females than expected survived the inoculation process and produced eggs in the post-infection vials. We consulted with Dr. Lazzaro for ideas to improve this, but were unable to increase the number of progeny produced. It has been previously documented that infection does reduce egg production (Hoffman et al. 1990), so this could partially explain the lower number of progeny that was counted compared to the control numbers. Having fewer overall surviving infected females also contributed to the lower number of progeny that was counted. So while we were still able to measure recombination rates in females that had been infected with *P. rettgeri* bacteria, we found that in our hands bacterial infection did not increase the number of recombinant offspring that were produced, in contradiction of what was reported by Singh et al. (2015).

Because our results did not show a reduction of recombination rates in the *cv-f* interval in the stocks that we needed to use to measure crossing over in mtd-RNAi females, we were unable to conduct the subsequent experiments to determine if *mtd* is required for increasing recombination in response to infection. However, our results do make it clear that *mtd* is required for normal levels of recombination to occur.

Future Directions

For future experiments, the lab will use genetic interaction experiments to determine if the way *mtd* influences the recombination rates goes through the IMD pathway. One approach to answer this question would be to perform RNAi of *mtd* as well as other genes in the IMD pathway; one hypothesis is that *mtd* sends the signal to increase recombination via the IMD pathway; therefore, if we knocked down other genes in the pathway, you might see a similar reduction in recombination rates. Alternatively, if reduced *mtd* levels causes the IMD pathway to reduce crossing over, then double RNAi of *mtd* with other pathway components (e.g. perform *mtd* and *relish* double knockdown) might cause recombination rates to be unchanged, as the signal would not be transduced. However, to test this requirement, RNAi constructs that work in the germline would be needed. Currently, there are no constructs against IMD pathway members in the VALIUM22 vector that is optimized for germline expression, which precludes this experiment. However, the TRiP project is currently in the process of making Valium 22 constructs against the IMD pathway members *rel, Fadd, dredd, imd* and *spc25.* By examining recombination rates in genotypes that have RNAi knockdown of those IMD pathway components, as well as double-knockdown of these genes with *mtd,* we will be able to see if *mtd* knockdown requires the IMD pathway to reduce recombination rates.

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