MATING-INDUCED CHANGES IN BEHAVIOR AND GENE EXPRESSION OF *CAENORHABDITIS REMANEI* FEMALES

by

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A THESIS

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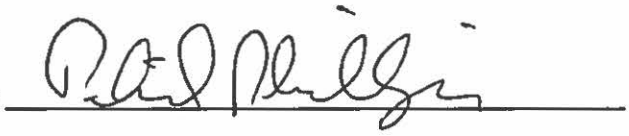
Approved: 

Dr. Patrick C. Phillips

Sexual reproduction plays a significant role in how many organisms pass on their genetic information by introducing diversity into the genomes of offspring. With sexual reproduction also comes the opportunity for conflict, however, as females and males counter-adapt to each other to fulfill their own fitness needs. This study uses the nematode *Caenorhabditis remanei* as a model organism to investigate female responses to mating at both the behavioral and genetic levels. Using behavioral assays and RNA-seq, we found that mating causes females to eat more and move less than their virgin counterparts. These adaptations are likely the result of a pathway in response to mating that confers a fitness benefit to these females.

Acknowledgements

I would like to first like to thank my mentor, Dr. Nadine Timmermeyer, for her expertise and guidance in this study. She worked with me tirelessly through every step of this process; this thesis would not have been possible without her. I would also like to extend my appreciation to Dr. Patrick Phillips for always providing the overarching vision that is absolutely paramount for a project like this – I always came out of our meetings inspired to strive even further with my work. I am privileged to have such excellent and knowledgeable advisors to help me through this process. Thanks also to the entire Phillips lab, whose sense of humor helped me through even the most stressful parts of my research, and to Anna, Eric, and Angela, who always willingly lent a hand when Nadine and I needed more labor. I also sincerely appreciate the support of my friends and family, who have continually provided a sympathetic ear and/or ice cream throughout my last year of work on this project.

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Introduction

Evolution: Shaping Life Around Us

Evolution is often associated with striking **adaptations** of an organism to its environment: long legs benefit a giraffe strolling across the plains, but for a mole living underground, long legs would be a hindrance. Evolution, the change in inherited traits in a **population** across generations, has created diversity at the genetic level which has translated to a wide spread of **phenotypes** across species. Evolution can occur across all biological levels: species, individual, and even molecular (Ridley, 2004). Natural selection is the means by which evolution occurs, and favors organisms with characteristics that lead to increased fitness (Ruse, 2009).

Adaptations are heritable traits that contribute to an organism's fitness, the amount of offspring an organism can produce in relation to other individuals of a population (Ruse, 2009). Fitness is defined this way because evolution occurs in the heritable traits contained via the genetic material that is passed on from parent to offspring. Fitness on an individual level is dependent on an organism's response to the environment, which includes members of the same species. While a certain phenotype translates to a certain level of fitness in a given environment and population, a **genotype** can result in multiple phenotypes because of changes in expression due to **epigenetic** modifications and processes like **transcriptional regulation** (Voet & Voet, 1995)**.** The ability to respond to environmental changes by modifying gene expression can potentially produce a phenotype with a higher fitness, which makes it an invaluable adaptation for any organism.

Sexual reproduction, or mating, is considered a significant adaptation in passing on genetic material. Asexual reproduction results in genetically identical offspring, but sexual reproduction introduces diversity into the genome, which can lead to a higher fitness (Ridley, 2004). Whatever the species-specific rituals are, the end product of mating is male and female gametes (sperm and egg) combining to form offspring (Ruse, 2009). Instead of an exact copy of either the mother or father, the offspring is now a combination of the two.

Mating is such an integral process that it has driven a number of adaptations that help ensure either the process of finding a mate or assure the fidelity of the mating. The peacock, for example, uses its ostentatious tail to attract females, and the bigger and more colorful the tail, the more likely he is to attract a mate (Ridley, 2003). He also competes with other males for females, and the bigger and better tail tends to win over the female. This tail, however, also makes the peacock more susceptible to predation. This illustrates the importance of the peacock's tail to mating: Even though it probably puts the peacock in danger, it is so effective at attracting a mate that this adaptation has persisted in the peacock genome (Ridley, 2003).

While competition occurs between males for females, sexual conflict can also arise between males and females because different strategies may be involved in maximizing fitness within a given sex. Generally speaking, females have a limited amount of eggs and males have excess sperm (Ridley, 2003). This leads to competition between males, which can drive adaptations like the peacock's tail, as well as some that are potentially harmful to females (Parker, 2006). The male bean-weevil, for example, has a spiky penis that allows it to better stay inside the female while mating (Figure 1).

While this is an advantage for the male, the spikes can cause physical harm to the female. Adaptations like these can lead to co-evolution, essentially an arms race between males and females (Parker, 2006). Females can adapt to male harm, which in the case of the bean-weevil, is the female adapting to kick the male during mating to minimize damage from the spikes (Rönn, Katvala, & Arnqvist, 2007). Males then have to adapt to this adaptation, which inflicts more harm on the female, leading the female to adapt again, and so on. This creates a cycle of males and females adapting to each other, with males attempting to assure their paternity and females trying to minimize harm (Parker, 2006; (Rönn et al., 2007).

Figure 1: The bean weevil penis

The bean weevil penis is covered in hard spikes and assists the male in staying inside the female during mating (Rönn et. Al, 2007).

This study aims to investigate adaptations in females in response to mating. The nematode *Caenorhabditis remanei* is an ideal model organism for answering questions surrounding these processes. This study focuses on mating-induced changes in female *C. remanei,* which are assumed to be the result of changing **gene expression** rather than

gene polymorphisms. To accomplish this, mated female and virgin female behavior and genetic expression will be compared directly. The change between the two states can be observed at the behavioral level and quantified at the genetic level using RNA sequencing. These results will allow us to analyze these responses and perhaps speculate why these adaptations exist.

From Genotype to Phenotype: A Journey in Molecular Biology

The Central Dogma of molecular biology describes the flow of sequence information from **DNA** to **RNA** to protein (Figure 2) (Voet & Voet, 1995). All of the information necessary to make a protein is encoded in the genome. The two relevant transfers of information in this process are transcription, DNA being copied into **mRNA,** and translation, proteins being synthesized from these transcripts (Voet & Voet, 1995). **RNA polymerase** transcribes DNA from its double stranded structure into single stranded **mRNA**. While DNA, housed in the **nucleus** of the cell, is very stable and its code well conserved, **eukaryotic** mRNA is easily degraded and extensively processed before being exported from the nucleus (Voet & Voet, 1995).

While RNA polymerase is transcribing the DNA into mRNA, a 5’ cap is added so the mRNA can be recognized by the ribosome, the cellular machinery that translates the information encoded by mRNA into protein (Voet & Voet, 1995). Additionally, a **poly-A tail** is added to the transcript, a feature important for protecting the mRNA from premature degradation as well as exporting the mRNA from the nucleus and translation (Voet & Voet, 1995). After transcription, splicing further modifies the mRNA by removing **introns** and leaving behind only **exons.** After transcription, the mRNA is

translated into protein. The ribosome reads **codons** encoded in the mRNA and uses this information to catalyze the formation of **peptide bonds** between amino acids, a process that eventually builds a **polypeptide chain**, the precursor to a full-fledged **protein** (Voet & Voet, 1995).

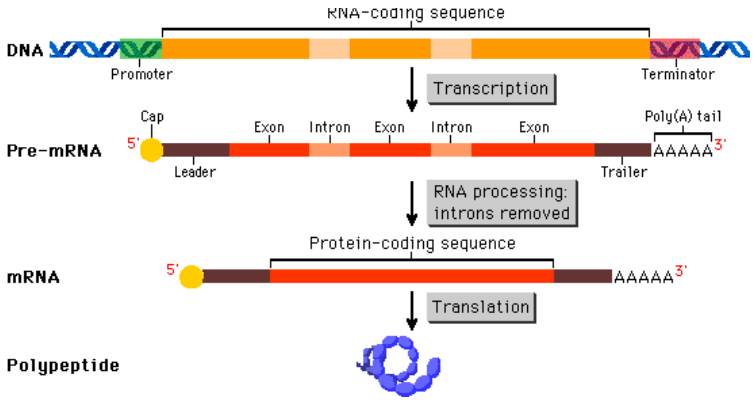


Figure 2: The Central Dogma of Molecular Biology

The process of going from DNA to proteins in eukaryotes. (http://www.phschool.com/science/biology_place/biocoach/transcription/mrnaeuk.html

Every addition of an amino acid takes an input of energy in the form of **GTP hydrolysis.** Considering this and the amount of machinery it takes to make a protein, protein synthesis is energetically very costly for a cell. For this reason, at both the transcriptional and translational levels there are checkpoints, proofreading, and stringent regulation to ensure these processes are performed accurately and that only the necessary products are made. The majority of the regulation occurs at the level of

transcription for a very simple reason: Why make the precursor for a protein if the protein is not needed?

Modulating output at the transcriptional level (how much RNA is made) can have an effect at the translational level (how much protein is made). This basic premise is utilized in organisms ranging from the simplest bacteria to higher order organisms like humans (Voet & Voet, 1995). The cell has a vast range of techniques it can employ to control transcription: **Transcription factors** can act as repressors and bind to the DNA and block RNA polymerase from initiating transcription or they can act as enhancers and promote the interaction between RNA polymerase and the target stretch of DNA. In eukaryotes, DNA itself can be remodeled so that RNA polymerase is unable to transcribe it (Voet & Voet, 1995). Causing a gene to be transcribed more is known as **upregulation** and causing it to be transcribed less is known as **downregulation.**

Stress responses in particular are characterized by these types of regulation. Without heat stress, for example, a small amount of RNA can be produced which translates to a small amount of the necessary proteins (Figure 3). With heat stress, however, more RNA can be transcribed which will result in more protein. This is a powerful tool because it allows for response to changes in environment (such as heat stress), while changing just one step -- how much transcription occurs -- has a downstream effect on how much protein is produced (Voet & Voet, 1995).

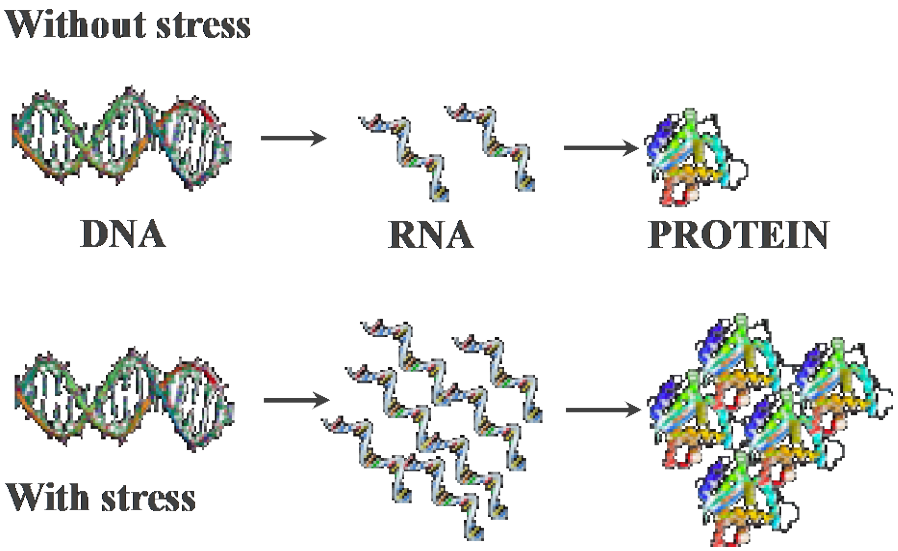


Figure 3: The power of transcriptional regulation

Regulation going from DNA to RNA (transcription) can lead to a differential output going from RNA to protein (translation). This particular example is of a stress response -- in the presence of stress, less RNA and therefore less protein is made. With stress, more RNA and more protein is made.

This process provides the basis for this study. This study hypothesizes that mating causes a change in gene expression in females, which in turn lead to observable changes in behavior. Just like the stress response example illustrated above, this would mean that modulation occurs at the transcriptional level. While it would be impossible to characterize all the proteins produced by the *C. remanei* female, because of recent technology, it is possible to simultaneously quantify the levels of RNA of tens of thousands of genes.

Overview of *Caenorhabditis remanei*

While the more famous relative of *C. remanei*, *C. elegans*, is hermaphroditic, *C. remanei* is gonochronistic, meaning that is has separate males and females. As a model organism, *C. remanei* retains the advantages of the *Caenorhabditis* genus, such as a well-investigated genome, clear body, and short generation time (three days), while also allowing researchers to study the dynamics of sexual selection and evolution (Kiontke & Sudhaus, 2006). *C. remanei* usually live in compost or woodland habitats and associate with snails or isopods in the wild (Kiontke & Sudhaus, 2006). In the lab, *C. remanei* are kept on agar plates and fed *Escherichia coli*. The strain used in this study, PX443, is a highly diverse strain made from crossing 26 different natural isolates from a single location. The genetic diversity of this strain makes it an excellent candidate for this study because it is close to what one might find in a natural habitat.

The *C. remanei* life cycle includes an embryonic stage, four larval stages (L1-L4) and adulthood. Each larval stage is separated by a molt, after which a new, stage-specific cuticle is made and the old one shed (Russell & Cassada, 1975). Adulthood is characterized by sexual maturity. In a lab setting, females will produce roughly 500 offspring in their two-week lifetime.

Mating in *C. remanei*

Females produce sex pheromones that attract males, but not vice versa (Chasnov, So, Chan, & Chow, 2007). These sex pheromones have potent effects on males — when plates containing females are in the vicinity of male-only plates, males often dry up on the walls of their plate because they attempt to escape and find females. This mate-

searching behavior is well characterized, and is influenced by both the female pheromone and the male's own nutritional status (Lipton, Kleemann, Ghosh, Lints, & Emmons, 2004). At sexual maturity, females start producing the pheromone and males start responding to it (Chasnov et al., 2007). The female's attractiveness is not a permanent state, however. Immediately after mating, females are less attractive to males, but they regain this attractiveness 12-18 hours post-mating (Chasnov et al., 2007). The female probably regulates this effect because pheromone production is energetically costly: she only produces the pheromone to attract males when she is sperm-depleted, and stops trying to attract males while sufficiently supplied with male gametes.

In order to mate, the male scans the female's body with its tail, which is equipped with specialized sensory receptors, until it locates the vulva (Barr & Garcia, 2006) (Figure 4). Once it finds the vulva, it releases a factor that rapidly immobilizes the female and also causes the vulva to widen, allowing for immediate insertion of the spicule (Garcia, LeBoeuf, & Koo, 2007)(Figure 5). The male then ejaculates its seminal fluid, and while it is removing the spicule from the female, also starts secreting a copulatory plug (Chasnov et al., 2007). While these mating plugs were originally thought to prevent the female from re-mating with other males, they have recently been shown to directly affect female fitness and allow them to produce more offspring (Timmermeyer et al., 2010). After females have mated, **serotonin** pathways have been shown to coordinate egg-laying and movement behavior (Hardaker, Singer, & Kerr, 2001).

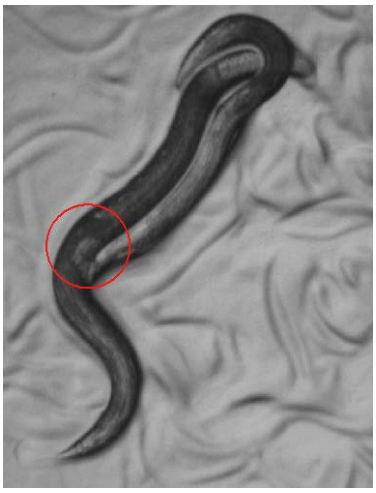


Figure 4: Male and female *C. remanei* mating

The female is on the left and the male on the right. The circled region indicates the male spicule inserted in the female's vulva. The white area in the female is the seminal fluid ejaculated by the male. Source: N. Timmermeyer

Figure 5: The male tail

The fan-like structure is used to scan the female, and the spicule (circled) is inserted into the female. This leads to the cascade of a events including paralysis of the female due the soporific inducer and ejaculation of the seminal fluid. Source: N. Timmermeyer

From pheromone secretion, to spicule insertion, to induction of the temporary female paralysis, almost every step in the mating process is more efficiently accomplished in *C. remanei* than either *C. briggsae* and *C. elegans,* its well investigated, hermaphroditic relatives (Chasnov et al., 2007; Garcia et al., 2007). It is speculated that this occurs because *C. remanei* is a separate-sex species and these nematodes therefore experience higher sexual selection pressures than species with selfing hermaphrodites (Garcia et al., 2007). Additionally, studies have shown that *C. remanei* have adapted to an optimum sex ratio of 50:50, as female fitness is highest when there is an equal number of males and females (see Figure 6) (Timmermeyer et al., 2014). Below this ratio, the females' egg production exceeds the amount of sperm it can receive, and above this, the physical harm caused by mating reduces the female's fitness (Timmermeyer et al., 2014) This principle will be exploited in our study to further compare effects after mating: a "super-mated treatment" was used to see if stressing the sex ratio exacerbates the changes seen after mating (Figure 7).

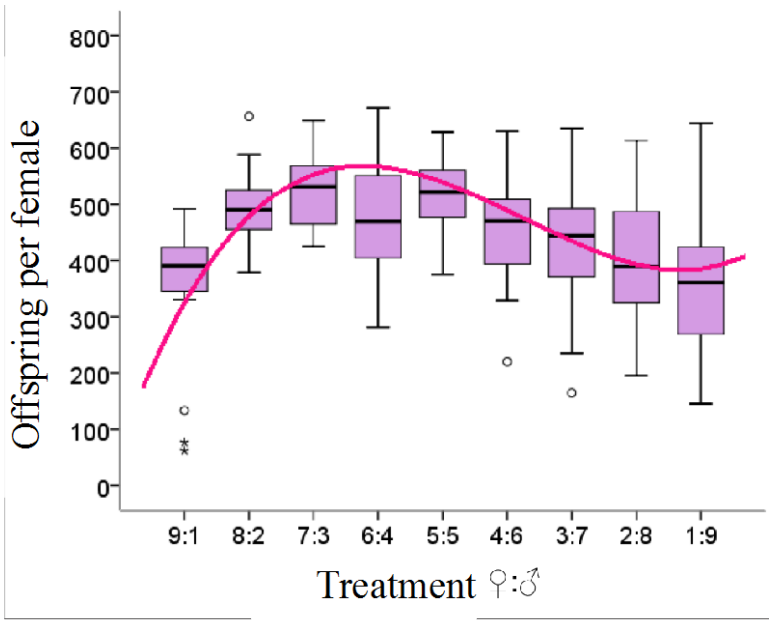


Figure 6: Comparing fitness as sex ratio changes

The above curve shows an optimum females fitness at around an equal sex ratio. Female fitness declines as the sex ratio tends towards more or less males. The fitness results with more males are probably caused by male harm to females. Source: N. Timmermeyer

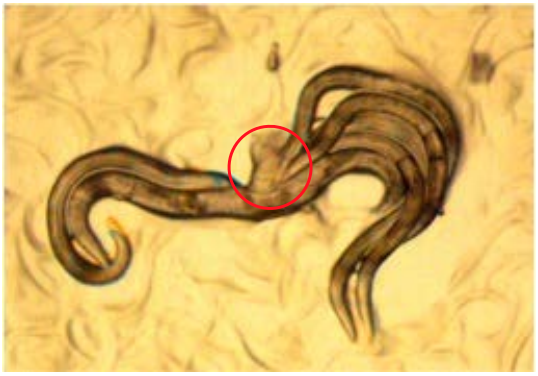


Figure 7: A super-mated female

The female (the middle, horizontal nematode) is plated with a 1:5 (female: male) sex ratio. This picture shows five males trying to mate with this female at once. The copulatory plug, indicated with the red circle, is also very obvious on this female. Source: N. Timmermeyer

Documented Male Harm in *Caenorhabditis* Species

Recent studies have shown that mating induces shrinking and death in *C. elegans* and *C. remanei* mothers. These studies have found that male sperm directly induces shrinking, which causes an increased susceptibility to **osmotic stress**, and that a component of seminal fluid leads to an important transcription–factor-mediated (**DAF-16**) lifespan decrease (Shi & Murphy, 2014). This is thought to create a high reproduction rate and low lifespan state in the females (Shi & Murphy, 2014). In a study focusing on lifespan shortening, the researchers identified male-secreted peptides that induced a gene expression profile which leads to a shortened lifespan (Maures et al., 2014).

Here, I test the hypothesis that mating induces changes in the behavior and the gene-expression profile of female *C. remanei* by comparing the behavior and RNA expression levels of mated and virgin females. I also use a “super-mated” treatment with increased male frequency to test whether exceeding the optimum mating ratio exacerbates the observed changes. Together, these approaches allow me to investigate mating-induced changes—and potential adaptive females responses—within this species for the first time.

Materials and Methods

Strain Use and Maintenance

T. Ahearne crossed the PX443 strain from 26 *C. remanei* Toronto natural isolates: PX390-PX410, PX417, PX418, PX420-PX422. A stock of PX443 aliquots is kept at –80 °C and thawed previous to the experiments (Sikkink, Reynolds, Ituarte, Cresko, & Phillips, 2014). After thawing and in preparation of the experiments, *C. remanei* were kept on 10 cm NGM Lite (Nematode Growth Medium, Lite; US Biological, Cat. # N1005) agar plates seeded with OP50 *E. coli* at 20°C (Stiernagle, 2006). The nematodes were chunked, which involves cutting out a small section of agar from a plate with nematodes and transferring the chunk to a plate without nematodes, every few days to avoid starvation (Stiernagle, 2006). The nematodes were also bleached, a procedure that only eggs survive, two days before each experiment to ensure that the nematodes were in the L4 stage by the time of the experiment (Stiernagle, 2006). In this study, virgin nematodes are isolated at the L4 stage, reached approximately 48 hours after hatching when hatched in liquid, to ensure that the females collected at this stage are virgins because they have not reached sexual maturity.

Pharyngeal Pumping Assay

Preparation and Treatments

Individuals were age synchronized using a sodium-hypochorite procedure ("hatch-off") in which adults are dissolved leaving only eggs behind. These eggs were plated onto a 10 cm agar plate seeded with 100µl of the OP50 strain of *E. coli*.

Following two days of development, 60 females in the L4 stage were placed onto separate 3 cm agar plates seeded with 20 µl of *E. coli* and assigned randomly to their treatments: virgin, mated, and super-mated. Each treatment consisted of 20 females. The next day, males were added to the necessary treatments in their respective ratios: 0 males for each virgin plate, 1 male for each mated plate, and 5 males for each super-mated plate (120 males total). The virgin treatments were only sham treated, which means the plate was open and closed while under the microscope.

Measuring pharyngeal pumping rates

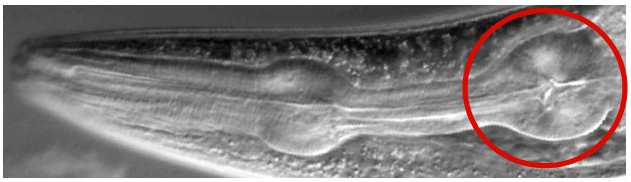


Figure 8: The nematode pharynx

The terminal bulb, which along with the corpus is contracted and relaxed during pharyngeal pumping, is indicated in the red circle. Source: http://elegans.som.vcu.edu/Worm_labs/Avery/Pictures/pharynx.gif

When the females were two-day old adults, pharyngeal pumping was examined. Pumping is defined as "one complete cycle of synchronous contraction and relaxation of the corpus and the terminal bulb" (Raizen, Song, Trojanowski, & You, 2012)(See Figure 8). The number of times the pharynx pumped was counted in a 30 second period. There was a 30-second period of rest, and the pharynx pumping was counted again. Each 30-second period of counting was considered one trial, and three trials were performed per nematode. The three trials were averaged for each nematode and these data were subjected to various statistical analyses.

Locomotion Assay

Preparation and Treatments

Nematodes in this assay were prepared and divided into treatments in the same way nematodes in the pharyngeal pumping assay were. We performed a pilot study with a limited number of replicates to establish this assay that was adapted from an assay in a previous study (Lipton et al., 2004). When nematodes crawl through the *E. coli*, some adheres to their bodies and they leave visible trails as they move on the plate. To better visualize this movement, all agar plates (preparation plates and experimental plates) were seeded with Green fluorescent protein (GFP) *E. coli* instead of the OP50 strain. GFP transforms blue light into green light that can be observed under a microscope using a set of filters that only allow the green light emitted by the GFP *E. coli* to be seen.

Assessment of Nematode Movement

Males were also included in this assay to establish a control with which to compare mated/super-mated and virgin female movement. Once all nematodes were two-day old adults, each female nematode (60 total) and 20 male nematodes were separated and placed near the edge of the agar (so the *E. coli* lawn was not disturbed during placement) on their own 3 cm plate seeded with GFP *E. coli*. 24 hours later, the nematodes were removed from the plates and the plates placed at 37°C to allow the *E. coli* to grow (explanation in Figure 9). 24 hours later, the *E. coli* on the plates was visualized by blue light exposure and photographed.

A crowd-sourcing method of rating the plates was used to assess the amount of

movement on each plate. I created three google forms with twenty plates each on them, and sent the forms to sixty people, resulting in 20 ratings per plate (Appendix A). Figure 10 shows the plates given as examples as a “1” or a “5”. JMP 9.0 was used to generate an average score for each plate and analyze the results.

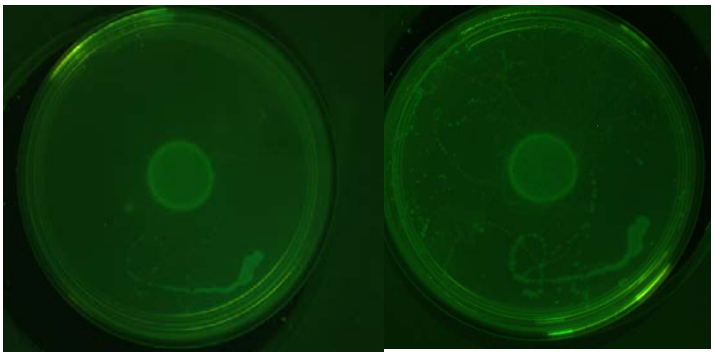


Figure 9: The importance of incubating plate at 37°C after nematode removal.

The plate on the left shows nematode tracks immediately after nematode removal, while the plate on the right shows the same plate 24 hours after incubation at 37°C. The plate on the right clearly shows more tracks that are only visible after the *E. coli* is allowed to grow for 24 hours.

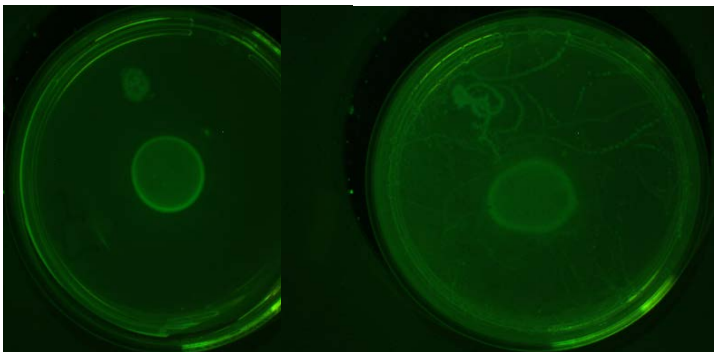


Figure 10: Examples given for movement ratings.

The left image is a “1” because the nematode did not move from where it was placed. The right image is a “5” because the nematode tracks cover the entire plate.

RNA-seq methods

Sorting/ Tissue Collection for RNA-seq

The first round of tissue collection was unsuccessful because sufficient tissue was not collected to prepare each sample for sequencing. 400 females per treatment (virgin, mated, super-mated) were picked by hand, which resulted in 1200 females total and 2400 males total. For the subsequent round of tissue collection for RNA sequencing, a combination of microfluidic sorting and hand-picking was used to collect nematodes. 1000 females per treatment were collected.

The Sorter

The sorter, developed in lab by J. North and N. Timmermeyer, is a polydimethylsiloxane (PDMS) microfluidic chip that uses computer-controlled valves to control the movement of nematodes through it. The medium itself is air permeable, which is important for reducing stress while sorting nematodes.

Sorting

The sorter is used to separate males from females both before and after treatments. Nematodes are inserted into the cathedral at the bottom of the chip, which leads to the parachute, labeled P in Figure 11. The “C” labels indicate a channel, where nematodes are able to flow through, and “V” indicates a valve. Opening and closing the valves control the flow of water through the channels, which in turn controls the movement of the nematodes from the bottom to one of the three top channels. Figure 12 shows the sorter with nematodes inside. To allow a nematode into the middle circle, valves 1 and

2 are opened. The narrow channel the nematode enters is the sorting channel -- If the nematode is female, valves 3 and 6 would be opened and the female sent to the left. If male, valves 3 and 4 would be opened and the male sent to the right. If the sex is undetermined, the valves 3 and 5 can be opened and the nematode flushed through the middle. The contents of the female and male channels feed into a conical tube where they are collected.

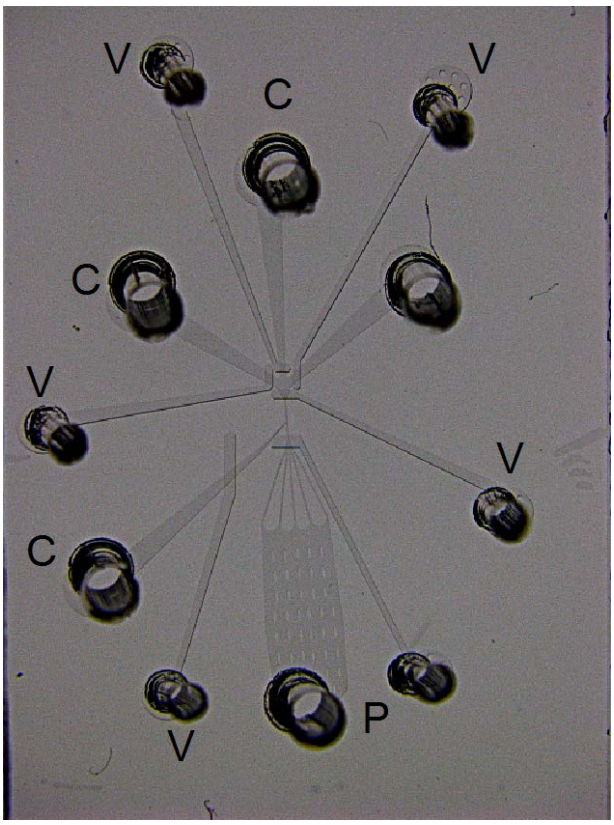


Figure 11: The sorter.

"C" labels indicate channels were water and nematodes can flow, and "V" indicates the valves that control this flow. The "P" is the parachute channel that leads to the sorting channel in the middle (circle in Figure 12). Source: N. Timmermeyer

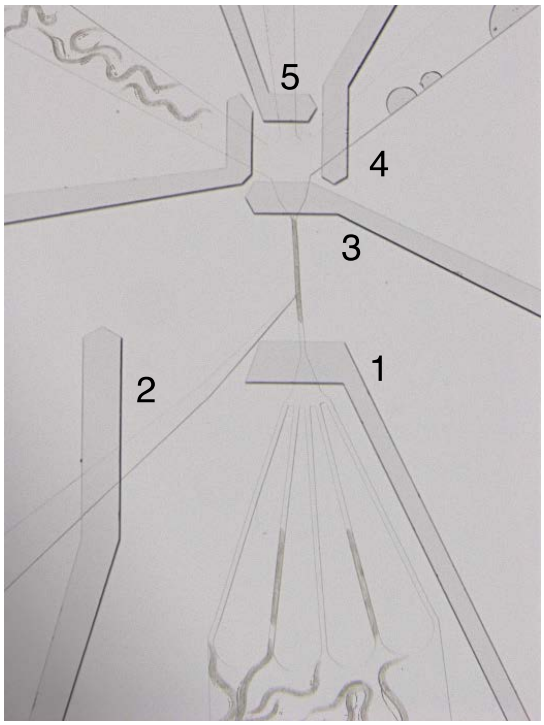


Figure 12: Sorter with nematodes

Nematodes are shown in the initial channels of the parachute as well as the circle decision channel and the male channel. Nematodes in the sorting channel are flushed to the right, center, or left depending on the sex. Source: N. Timmermeyer

Females also had to be sorted from males after their treatments (explained in Mating Treatments), which posed a problem -- adult females, especially those that have mated and have copulatory plugs and/or eggs, are much larger than L4 females. Adult females would block the channels in the current sorter, and we therefore had to develop a larger sorter for the adult females post-treatment. In order for the sorter to function correctly, the channels had to be the exact size of the largest adult female. If they were too big, multiple nematodes could fit into the sorting channel, and if they were too

small, nothing would flow through. We developed a sizing chip to estimate how big the sorting channels would need to be to accommodate the larger females (Figure 13).

Every five microns, the channel becomes five microns narrower -- where the nematode ended up in the channel therefore determined how big it was and gave us an estimate for the minimum size of the channel. Because the nematodes are round, height of the channel also had to be taken into consideration, and the final design for the sorter used channels that were 50 microns high and 65 microns wide.

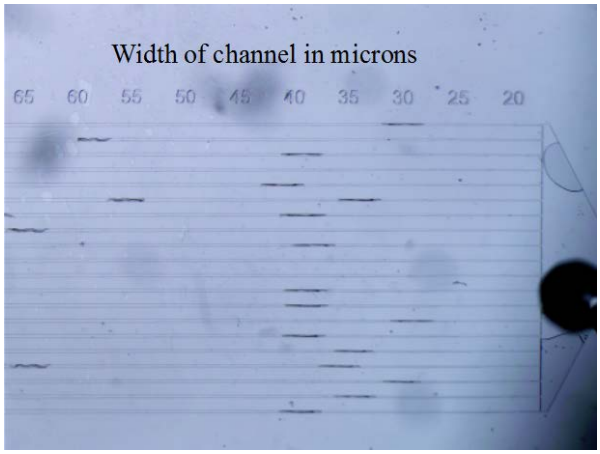


Figure 13: Sizing chip

Nematodes were pushed through the sizing chip until they could not move any farther through their channel. This gave us an estimate of the average size of adult nematodes. Adult females were found to be much larger than L4s, so this information helped determine the necessary size of channels for a larger sorter.

Mating Treatments and Preparation

Nematodes were synchronized and plated on ten 10cm seeded *E. coli* plates. After two days of development, nematodes were washed off with **S-Basal** into a 15 mL conical and prepared for sorting. About 30,000 nematodes were prepared for sorting to

ensure we had enough for the treatments and to account for error in sorting. 500 females were collected per treatment, which resulted in a total of 1500 females and 2000 males sorted. An additional 1500 females were hand-picked, separated, and assigned to treatments. The virgin treatment contained five females per plate and no males, mated treatment contained five females and five males, and super-mated treatment contained five females and fifteen males. The super-mated sex ratio was adjusted from the previous assays (1:3 instead of 1:5) because of workload restrictions. Females were pipetted onto the *E. coli* seeded 3 cm agar plates under the microscope (to see how many were being pipetted) and males were added to the necessary treatments. After 48 hours, the nematodes were sorted again but with the larger sorter, and separated by treatment. Only the females were used for sequencing, and were separated from males within their respective treatments. The females were transferred into 1.5 mL tubes and prepared for sequencing, which is detailed below.

RNA-seq library preparation for Illumina sequencing in C. remanei

This protocol is adapted from a protocol that prepares L1 *C. remanei* for sequencing (Sikkink, 2013). Following separation by treatment group, the nematodes were allowed to settle to the bottom of the collection conical and approximately 1mL of was transferred into a 1.5 mL tube. The nematodes that had been hand-picked rather than sorted were washed off from their plates with approximately 4 mL of S-Basal and transferred to a conical, allowed to settle at the bottom, and transferred. Treatment tubes were centrifuged and their volume reduced to about 30 ul. 1 mL of TRIzol (Invitrogen, Cat. # 15596- 026) was then added to stabilize the RNA. The tubes were then flash-

frozen in liquid nitrogen and subjected to Freeze-Cracking**,** which alternates flash-freezing in liquid nitrogen and thawing in a warm water bath to mechanically remove the cuticle of the nematode and allow access to the RNA (Portman, 2006). Total RNA was extracted using TRIzol extraction (Portman, 2006) and confirmed via gel electrophoresis of 1 ul of each sample on 1.1% agarose (1x TAE) (Figure 14). Messenger RNA was isolated from the total RNA using a Dynabead Oligo(dT) $_{25}$ kit (Life Technologies, Cat. #61011) and fragmented using an RNA fragmentation kit (Ambion, Cat. # AM8740)**.**

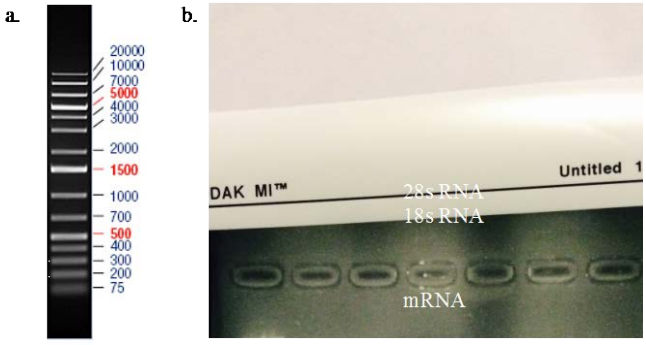


Figure 14: A) 1kb plus DNA ladder B) Gel electrophoresis of total RNA

A) This an annotated version of the ladder (Thermo Scientific, Cat # SM1331) applied to the far left lane in Figure 14B. The bands provide a reference for determining the size of the RNAs separated on the gel. B) The two brightest bands in each lane are 28S and 16S ribosomal RNA (rRNA) (Ellis, R.E., Sulston, J.E., Coulson, 1986; Portman, 2006). The smears near the bottom are the mRNA – the mRNA migrates farther down the gel because it is much smaller than the rRNA.

cDNA was generated from the mRNA using **reverse transcriptase** Superscript III (Invitrogen, Cat. # 18080093) and *E. coli* DNA Polymerase I (NEB, Cat. # M0209L), followed by purification with the DNA Clean & Concentrator Kit (Zymo, Cat. # D4030) to eliminate excess reagents and unwanted products before continuing to the

next step. cDNAs were blunt end ligated with the addition of an A-overhang. Following ligation of a unique barcode adapter, the cDNA was amplified *via* PCR (Polymerase Chain Reaction) and loaded onto a 1% agarose gel (0.5x TBE) for size fractionation. cDNA in the 200-600 bp range was excised from the gel and the QIAquick Gel Extraction Kit (Qiagen, Cat. # 28704), and MinElute columns were used to extract and purify the cDNA. Following quantification with **Qubit** fluorometer, the nanomolarity was estimated based on the fragment size. An equal weight of all samples was then pooled together and diluted to a 10nM concentration. The libraries were sequenced from a single end with read lengths of 100 base pairs using Illumina Hi-Seq.

Analysis of RNA-seq Data

The raw data from RNA-seq are sequences of fragmented mRNA transcripts. In order for this data to be intelligible, and to acquire information about which genes are being expressed and to what degree, there are a few necessary steps. This general protocol is adapted from another study that used RNA-seq to characterize changes (Sikkink et al., 2014). After some initial processing, the data will be aligned to the *C. remanei* genome using the software program *GSNAP*. This step will tell us which genes are being expressed. After alignment, transcript levels are initially counted with the HTSeq package in *Python*. HTseq is used as a filtration step before the final processing. Finally, DESeq, an *R* package, is used to normalize reads to the effective library size, and uses statistical analysis to determine differential expression. This final step will allow for the direct comparison between gene expression levels of mated, super-mated, and virgin females.

Results

Pharyngeal Pumping Assay: Mated and super-mated females take up more food in a given period than virgin females

0 –

Figure 15: Average pharyngeal pumping rates per treatment.

Virgin: Median rate of 25 times/ 30 seconds. Mated: Median rate of 123 pumps/30 seconds. Super-mated: Median rate of 117 pumps/ second. Box = 25th and 75th percentiles, bars = min and max values. DF = 45, Adjusted R squared = 0.77, F=75.1, $p<0.001$. Tukey Kramer post-hoc comparisons (shown with letters above) show that virgin pumping rates are statistically different from both mated and super- mated pumping rates, but mated and super-mated pumping rates do not differ.

This assay tested the hypothesis that mating causes females to exhibit more pharyngeal pumping behavior than their virgin counterparts. Pumping rates for mated females, super-mated females, and virgin females were observed and compared. The median pumping rate for a virgin female was 25 pumps/30 seconds, approximately 123 pumps/30 seconds in mated females, and 117 pumps/30 seconds in super-mated females (Figure 15). It is clear that virgins have a lower rate of pharyngeal pumping: while the

mated and super-mated nematodes tended to pump their pharynx at a constant rate, the virgins pumped more sporadically. A subsequent trial with increased sample sizes focused solely on mated and super-mated individuals did not reveal any additional differences between these treatments (Figure 16). Overall, these results demonstrate that after mating, females exhibit more pharyngeal pumping behavior, which allows them to take up more food. The ratio of males:females during the mating period does not seem to affect food uptake

Figure 16: Average pharyngeal pumping rates/ treatment: Mated and super-mated only. Mated: Median rate of 112 pumps/30 seconds. Super-mated: Median rate of 115 pumps/ second. These results suggest that the difference in pumping rates between the two treatments is not significant (two sample t-test, t ratio = -0.52, p> |t| = 0.6042.)

Locomotion Assay: Virgins females move more than mated or super-mated females

This assay tested the hypothesis that mating causes females to move less than their virgin counterparts. During the 24 hours the worms spent on the experimental

plate, three males and one super- mated female crawled up the walls and died, so were removed from subsequent analysis. Male plates had an average movement score of 4.2 ± 0.53, virgin plates a score of 3.4 ± 0.56, mated female plates a score of 2.4 ± 0.51, and super-mated female plates a score of 2.2 ± 0.52 (Appendix A). The fact that the standard deviations are small and consistent across the treatments suggests that this method of ratings plates is fairly reliable (For raw data see Appendix A). The movement scores show that while mated and super-mated movement is not significantly different, virgin movement and male movement are significantly different from both mated and super-mated movement (Figure 17). Virgin movement is also different from male movement, but mean comparison using Tukey-Kramer HSD post-hoc analysis shows that it is less dissimilar to male movement (difference = 0.86) than either mated movement (difference = 0.94) or super-mated movement (difference = 1.18). While pharyngeal pumping was not assayed in males, the results of the locomotion assay corroborate the results of the pharyngeal pumping assay in that there is no behavioral difference observed between mated and super-mated females but there is a difference between virgins and mated or super-mated females. Example plates of each treatment are shown in Figure 18.

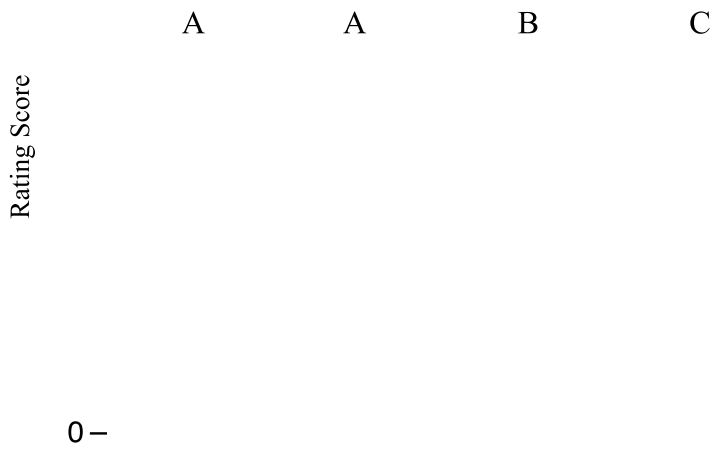


Figure 17. Movement rating scores from locomotion assay.

Median mated score: 2.5. Median super-mated score: 2.4. Median virgin score: 3.3. Median male score: 4.2. Anova analysis gave an adjusted R2 value of 0.52, DF =58, F Ratio = 21.6, $p<0.0001$. Tukey-Kramer post-hoc comparisons (indicated with letters above boxes) show that mated and super mated scores are not significantly different from each other, but that virgin and male scores are different from all other values.

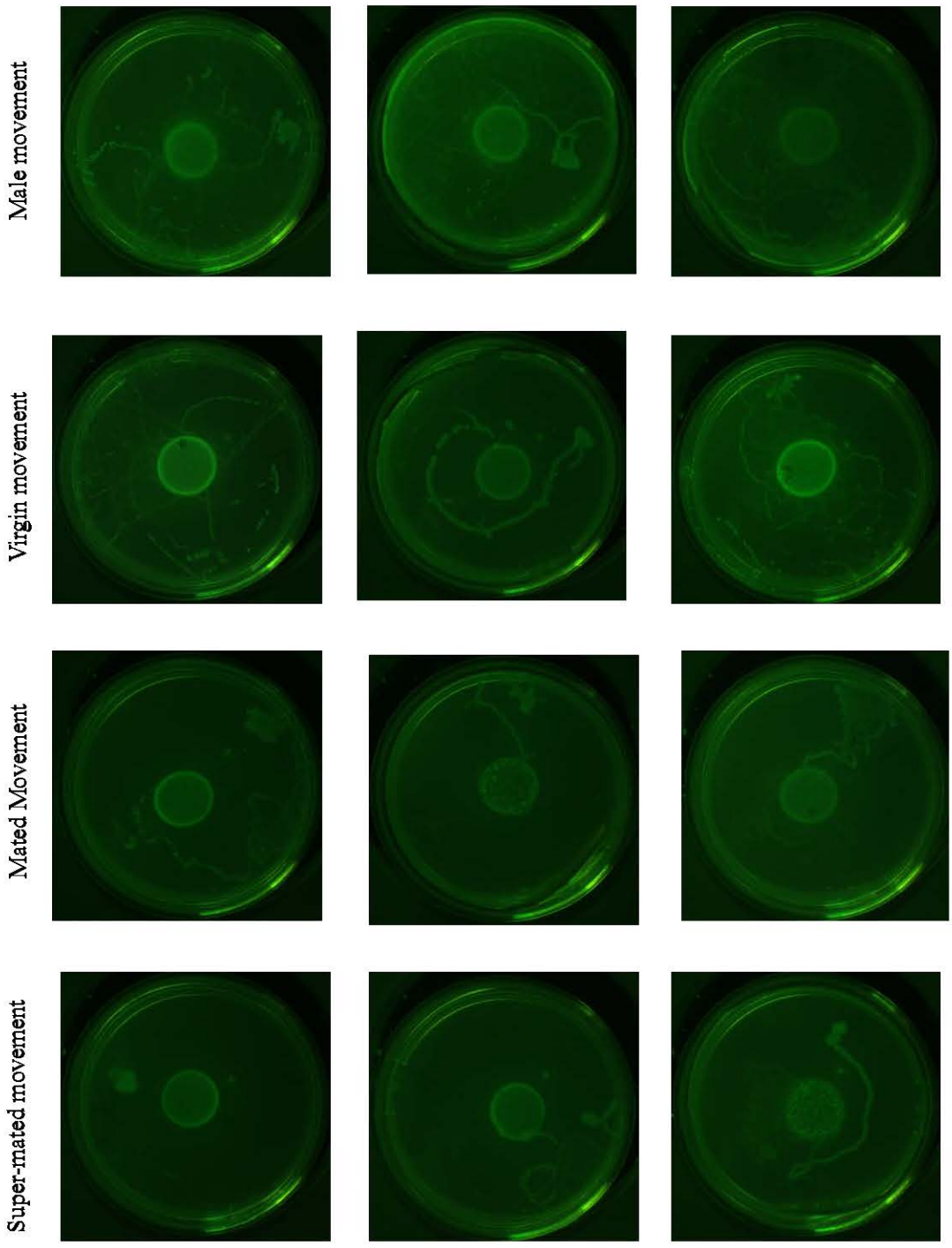


Figure 18: Example nematode trails from each treatment: Male, virgin female, mated female, and super-mated female.

Mated and super-mated females displayed the same amount of movement on their plates while males and virgins moved more.

RNA-seq results

At the time of this draft, the sequencing results are still pending.

Discussion

While the results of the sequencing experiment have yet to be obtained, those from the pharyngeal pumping and locomotion assay support the hypothesis that mating induces changes in female *C. remanei*. Both of these changes, increased feeding and decreased movement, have an impact on energy considerations for the female: mated females coordinate a simultaneous increase in energy input and decrease in energy output. Although not the focus of previous studies, it can be assumed that after mating females have increased energy demands, chiefly in producing and expelling eggs. Behavioral changes that conserve energy therefore likely confer some fitness advantages for the female, but this may be at the price of its own health. Multiple studies in *C. elegans* have demonstrated that decreased caloric uptake is correlated with a longer lifespan, which means that increased pharyngeal pumping after mating could be contributing to the decreased the lifespan of the female nematode (Bergamini, Cavallini, Donati, & Gori, 2003; Lakowski & Hekimi, 1998; Lant & Storey, 2010; Narasimhan, Yen, & Tissenbaum, 2009).

How does a response to mating that potentially decreases lifespan contribute to the female fitness strategy? Previous research has demonstrated that oocyte quality decreases with age (Andux & Ellis, 2008). Therefore, females may have evolved to induce metabolic changes that result in the production of as many oocytes as possible early in life, even if this reduces lifespan, because the oocytes produced later are less likely to be viable. Though this might seem counterintuitive, producing many viable oocytes in a short period of time could yield a greater fitness for the female than

producing fewer oocytes that decrease in quality over time.

Although it has been demonstrated that sex ratios that deviate from approximately 50:50 results in decreased fitness for the female, there were no significant differences in behavior between mated (1:1 sex ratio) and super-mated females (5:1 male to female sex ratio) (Timmermeyer, 2014). When there were more males than females, this decreased fitness could have been the result of male harm that physically damaged the females and impaired their ability to produce and lay eggs (Figure 6). From these results, we predicted a 5:1 male to female ratio to also impact the changes seen after mating. Although this was not observed behaviorally, the RNA-seq results could show differences in gene expression between the mated and super-mated. If this is not shown, it could be possible that females have multiple pathways in response to mating: one that is induced by default after mating, and another that is induced when mating conditions become too stressful. This suggests a possible separation between the responses to harm caused by mating and the response to mating itself. The harm pathway could be a more general response to physical damage, while the pathway in response to mating is specific to mating.

Another interesting result was that males and virgin females both moved significantly more than either mated or super-mated females, but virgin females still moved less than males. A possible cause of this difference is that females, as discussed previously, produce a pheromone that attracts males, but not vice versa (Chasnov et al., 2007). Males are able to sense this pheromone even when separated from females. Therefore while both might display mate-searching behavior, this behavior is expressed more strongly in males because they are driven by their attraction to this pheromone. It

is also possible that males and females express the genes necessary for locomotion differently, regardless of mating state, and this is enough to explain the differences in amount of movement between males and virgin females.

While behavior can be observed and quantified, such complex behavioral changes are likely modulated by a host of different regulatory elements that cannot be outwardly observed. RNA-seq is therefore employed to elucidate these regulatory networks, as well as other molecular changes that could be occurring in the mated female. Based on previous studies, there are a couple results of RNA-seq that we can predict with some certainty: I would expect my gene expression profile to corroborate the profile seen with mating-induced lifespan shortening (Maures et al., 2014). Additionally, because serotonin pathways have been implicated in egg-laying and locomotory behavior in *C. elegans* mothers, I would expect to see changes in expression of other factors in this pathway between virgins and mated females (Hardaker et al., 2001).

Mating-induced changes in females have only recently being studied, but responses to oxidative, starvation, heat, and pathogenic stress have been fairly well characterized in *C. elegans*, a close relative of *C. remanei*. While all the molecular mechanisms are not fully known, there are a few pathways that are well conserved, utilized consistently and are considered master regulators of stress response. It has been demonstrated that *C. remanei* males can harm to females during mating: they ostensibly damage the cuticle with their spicule and paralyze them for a few minutes while mating, both of which could be harmful and may contribute to reducing female lifespan. This damage probably causes stress to the female, and because the known stress pathways overlap so frequently, it possible that the female response to mating is regulated by the

same master stress regulators that also respond to environmental factors like oxidative stress, heat stress, or attack by pathogens. Using the paradigm of mating-induced changes as the result of an existing or novel stress response and pathway, it is possible to make some predictions about the results of RNA-seq based on previous stress response experiments.

General stress responses

There are a few pathways implicated in **hypometabolic** changes in response to stress, including Insulin/Insulin-like growth factor signaling (IIS), Transforming Growth Factor B (TGF-B) signaling and Target of Rapamycin (TOR) signaling (Lant & Storey, 2010). These involve, among other things, decreasing food intake in response to stress, which as mentioned previously, confers increased longevity to the nematode (Bergamini et al., 2003; Lakowski & Hekimi, 1998). It is intriguing to see an opposite response in females after mating -- this study observed that mated and super-mated females eat more in a given time period than their virgin counterparts. This could mean that whatever network is modulating increased food intake in females after mating has to suppress the networks that normally respond to stress with decreased food intake. In terms of what this signifies for RNA-seq data, because the above regulatory networks have an opposite effect on food intake than mating appears to have, I would expect an opposite expression profile in the genes regulated by these networks in mated females compared to nematodes who have been exposed to other types of stress. Genes that are normally downregulated in response to stress would be upregulated in mated females,

and vice versa. Considering that these networks exist to minimize damage from stress, it is interesting to consider the implications of a regulatory network that would directly oppose that, favoring fitness over the health of the female.

A couple transcription factors have also been shown to be necessary for multiple pathways, making them **pleiotropic**: DAF-16/FOXO has been demonstrated as necessary for heat shock response as well has UV resistance, oxidative resistance, and hypertonic resistance, and SLR-2/JMC-1 responds to multiple stress stimuli, including heat shock, osmotic stress, oxidative stress, and ethanol stress (Hsu, Murphy, & Kenyon, 2003; Johnson et al., 2000; Kirienko & Fay, 2010; McElwee, Schuster, Blanc, Thomas, & Gems, 2004; Narasimhan et al., 2009). Because these are involved in pathways for so many different types of stress, it is entirely possible that these are also implicated in the female response to mating stress. It would be interesting to see, if either of these transcription factors regulates the response to mating, if the response to mating stress is more similar to some responses over others. These transcription factors are also interesting in that they are responsible for producing resistance to stress, and it would be worth observing whether a similar resistance to mating stress could be produced *via* these factors.

Epidermal Damage

While not directly studied, it is likely that spicule insertion can damage the female cuticle and epidermis around the vulva. Females tend to mate more than once, making it possible that the harm caused by mating triggers a pathway that is responsible for

damage repair to prepare the vulva for additional matings. Previous studies have probed the coordinated responses to epidermal wounding, and have found many regulators of this process. Even though mammals, insects, and nematodes have vastly different epidermal structures, studies suggest that many wound healing pathways are evolutionarily conserved (Schäfer & Werner, 2007). In both mice and *Drosophila*, the activation of JNK and AP-1 transcription factors has a central role in mediating cell motility at the wound site and Grainyhead transcription factors are required for epidermal barrier repair (Jane, Ting, & Cunningham, 2005; Moussian & Uv, 2005; Schäfer & Werner, 2007). If these are conserved pathways, it is likely that these same transcription factors would be upregulated in female *C. remanei* only after mating, because virgins are less likely to encounter the same kind of damage.

Studies in *C. elegans* have discovered many pathways in response to damage from sources like lasers or needles or bacterial or fungal infections. DAPK-1, originally thought to be a regulator in cell death, was recently shown to have an important negative regulator of innate immune response in epidermal wounds (Tong et al., 2009). Interestingly, *dapk-1* mutants were found to upregulate among other genes, the same epidermal AMP genes that are upregulated by infection with *D. conispora* (Tong et al., 2009). *D. conispora* is an obligate parasitic fungus that associates with *C. elegans* and ultimately penetrates the cuticle by a combination of enzymic softening and mechanical force (Dijksterhuis, Veenhuis, & Harder, 1990). A study found that there were 275 genes commonly downregulated by *D. conispora* and *Harposporium*, another fungus, and nine genes were commonly downregulated by *Harposporium* and three other bacteria (Engelmann et al., 2011). All of the mentioned changes in gene expression

would be interesting to look for in the female gene expression response to mating, especially those that are commonly affected by different sources (i.e. bacterium and fungus, wound and fungus) because those suggest the existence of network that responds to general stress. However, it is important to remember that males probably cause less harm to the females than a parasitic infection -- damaging the female too much would reduce the male's own fitness if the female is unable to produce or lay eggs because of the harm caused to her.

If not transcriptional regulation, then what?

Transcriptional regulation is a powerful tool for modifying a phenotype in response to environmental changes, but there are other methods an organism can achieve this as well, chiefly neuronal or hormonal responses. While the results of these responses might be detected by RNA-seq, it is possible that the regulators of these responses will not be if they are in the form of neurotransmitters or hormones. For example, an estrogen receptor in C. *elegans* has been identified, and exposure to estrogen can change fecundity rate and number of germ cells (Mimoto et al., 2007). As mentioned previously, male food-leaving behavior is regulated by serotonin and IIS pathways in response to female pheromone (Hardaker et al., 2001; Lipton et al., 2004). On the neuronal side, the thermosensory neurons have been shown to initiate the heat response cascade in *C. elegans* (Prahlad, Cornelius, & Morimoto, 2012). Finally, Major Sperm Protein (MSP), which is responsible for sperm motility in *Caenorhabditis* species, has an important role in stimulating oocyte maturation even before the sperm meets the oocyte (Kuwabara, 2003). This represents a very powerful cell-to-cell

signaling pathway. While the effects on the oocyte have been investigated, MSP's effect on host maternal cells has not, and would be worth investigating.

Future Directions

Although the absence of the results of RNA-seq makes it difficult to predict what specific genes or molecules to study, there are still many other questions to be answered about mating-induced changes in females. It was interesting to observe that virgin females moves less than males, and it was postulated earlier that this is because males respond to the female-produced pheromone. This could be tested by exposing males to females that are unable to produce the pheromone, and comparing the amount of movement to movement of males who are exposed to normal females. Additionally, it was hypothesized in this study that super-mated females could exhibit exacerbated versions of mated-female behavior, but this was not demonstrated in the locomotion and pharyngeal pumping assays. However, it would be interesting to see if other effects after mating, such as the female cessation of pheromone production after mating, is affected by the ratio of males to females (Chasnov et al., 2007). Additionally, in a recent study, maternal environment was shown to influence resistance to heat stress in offspring (Sikkink et al., 2014). If mating causes a similar stress response, it could be possible that females who are exposed to more mating stress (super-mated females) influence the ability of their offspring to respond to that same mating stress.

As hinted at earlier, a major target of research after this study will be to elucidate the molecular mechanisms behind the regulatory networks induced by mating. MSP

would be a viable candidate for study because it is a direct product of mating and has already been shown to have cell-to-cell signaling capabilities. Non-coding RNAs represent a relatively new field of study of non-protein regulators of transcription that could also have a role in regulating mating-induced responses: Micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs) have both been discovered in *C. elegans*, with some miRNAs being linked to developmental arrest while lncRNA function is still being discovered (Ahmed et al., 2013; Nam & Bartel, 2012).

Conclusion

Through behavioral assays, this study identified changes in females caused by mating -- mated females were shown to eat more and move less than virgins. Other possible responses to mating such as changing gene expression, as well as the networks that regulate these changes, have yet to be elucidated, but hopefully the results of RNA-seq will provide insight into these changes. The investigated responses to mating likely represent a conserved pathway that has maintained a female response to mating that optimizes her own fitness. This might come at a cost, however, because the observed behavior associated with mating-induced changes could contribute to the shortened lifespan seen in mated females. This could be a part of the female fitness strategy: because oocyte quality decreases over time, the female might optimize fitness by producing many oocytes quickly -- even if the changes necessary to produce these oocytes decrease her lifespan -- because earlier oocytes are more likely to be viable than oocytes she can produce later.

Comparing our results to those of other studies, females may have adapted multiple response pathways to mating: one pathway works to maximize fitness at the cost of female health while the other, induced only under the most stressful conditions, favors health over fitness. We postulate that at least some aspects of mating are stressful to females, but the known stress responses have the complete opposite effect on metabolism. If mating has harmful effects on the female, which it probably does, how have females adapted to suppress the body's normal stress responses and induce changes necessary to produce offspring?

These types of questions can never be answered in a single study, but they provide the basis for hundreds of other studies. If relatively simple organisms can modulate such complex responses, then the changes that occur in more complex organisms, such as humans, are likely to be even more complicated. However, because comparable regulatory networks are conserved between organisms like fruit flies and mice, it is also possible that we would discover many similarities in the regulatory networks that control the mating-induced responses in other organisms. After all, while these changes may manifest differently, males and females across all species have had to adapt to each other to optimize their own fitness, and it is likely that these adaptations overlap somewhere.

Glossary

Adaptation: An inherited characteristic of an organism that increases its fitness.

cDNA: Literally "copy DNA," cDNA is DNA made from reverse transcribing mRNA. It is
complementary to the given mRNA and differs from DNA in that in only includes exons.

Codon: The unit of genetic code in DNA and RNA that is a sequence of three nucleotides.

DAF-16: An important transcription factor in *C. elegans* that works via the IIS pathway.

DNA: Deoxyribonucleic acid is a self-replicating material that carries genetic information.

Downregulation: The decrease in expression of either a single gene or multiple genes.

Epigenetic: Translating to "above the gene," epigenetic changes refer to nongenetic influences on gene expression.

Enzyme: A protein that speeds up a reaction.

Eukaryote: An organism that has a nucleus and other membrane-bound organelles within its cells.

Exon: A region of DNA that encodes information for synthesizing a protein. Exons are spliced together after transcription.

Freeze-cracking: A process that alternates freezing in liquid nitrogen and thawing in a hot water bath to mechanically remove the cuticle from a nematode.

Gel electrophoresis: A technique that separates mixtures of DNA, RNA, or protein by the charge and size.

Gene expression: A process in which information from a gene is used to synthesize a functional gene product, which is usually a gene.

Gene polymorphisms: A variation in a DNA sequence, usually a mutation that changes a single base pair.

Genotype: The genetic makeup of an organism.

GTP hydrolysis: A process that creates energy for the cell. One of the phosphodiester bonds on the GTP is broken, which releases energy and leaves GDP and a free phosphate group.

Hatch-off: A procedure that uses a sodium hypochlorite solution to synchronize a nematode population. The solution dissolves any unhatched nematodes and leaves behind only eggs. These eggs are then either hatched in liquid, such as S-Basal, or pipetted onto an agar plate seeded with *E. coli*

Hypometabolic: A state in which an organism has reduced metabolic function.

Introns: The noncoding region of DNA that is spliced out after transcription.
mRNA: Messenger RNA is a family of RNA molecules that is responsible for carry genetic information from the DNA. mRNA contains amino acid sequences that are translated into protein by the ribosome.

Nucleus: The usually double-membrane bound organelle that houses genetic material in the form of DNA.

Nucleotide: A compound consisting of a nucleoside (a base plus a sugar) linked to a phosphate group. The basic structural unit of nucleic acids.

Osmotic stress: Stress that is a result of a sudden change in the solute concentration of a cell.

Oxidative stress: Stress that results from exposure to reactive oxygen species known as free radicals.

Peptide bonds: A chemical bond formed between a carboxyl group of one molecule and the amino group of another molecule. These bonds are found between amino acids, the building blocks for peptides.

Phenotypes: The observable (usually physical) characteristics of an organism resulting from the interaction of its genotype with the environment.

Pleiotropic: Describes a gene that can affect multiple, seemingly unrelated traits.
Poly-A tail: A long chain of adenine nucleotides added to mRNA that aids with exportation of the mRNA and confers stability to the molecule.

Polypeptide chain: A short chain of peptides made from amino acids; precursor to a protein

Population: The sum of organisms in the same species who live in the same area and can interbreed.

Qubit:A fluorometer than can be used to measure the amount of DNA, RNA, or protein, in a given sample. Prior to using Qubit, target-molecule specific fluorescent dyes are bound to the molecule, and the fluorometer measure the amount of fluorescence.

Reverse transcriptase: An enzyme that catalyzes the formation of cDNA from an mRNA template.

RNA: Ribonucleic acid has varying roles in the cell, which includes carrying genetic information from DNA, catalyzing various reactions, and sometimes regulating processes like transcription. Some viruses encode their genetic information in RNA rather than DNA.

RNA polymerase: An enzyme that catalyzes the production of RNA from a DNA template.

S-Basal: A liquid medium that can support nematode growth.

Serotonin: A compound that acts as a neurotransmitter in many organisms.

Transcription factors: A general term for a small molecule that can either activate or repress transcription, generally by binding directly to DNA

Transcriptional regulation: The process by which gene expression is controlled, as well as the fidelity of transcription itself.

Upregulation: An increase in gene expression in either one gene or multiple genes.

Appendix A

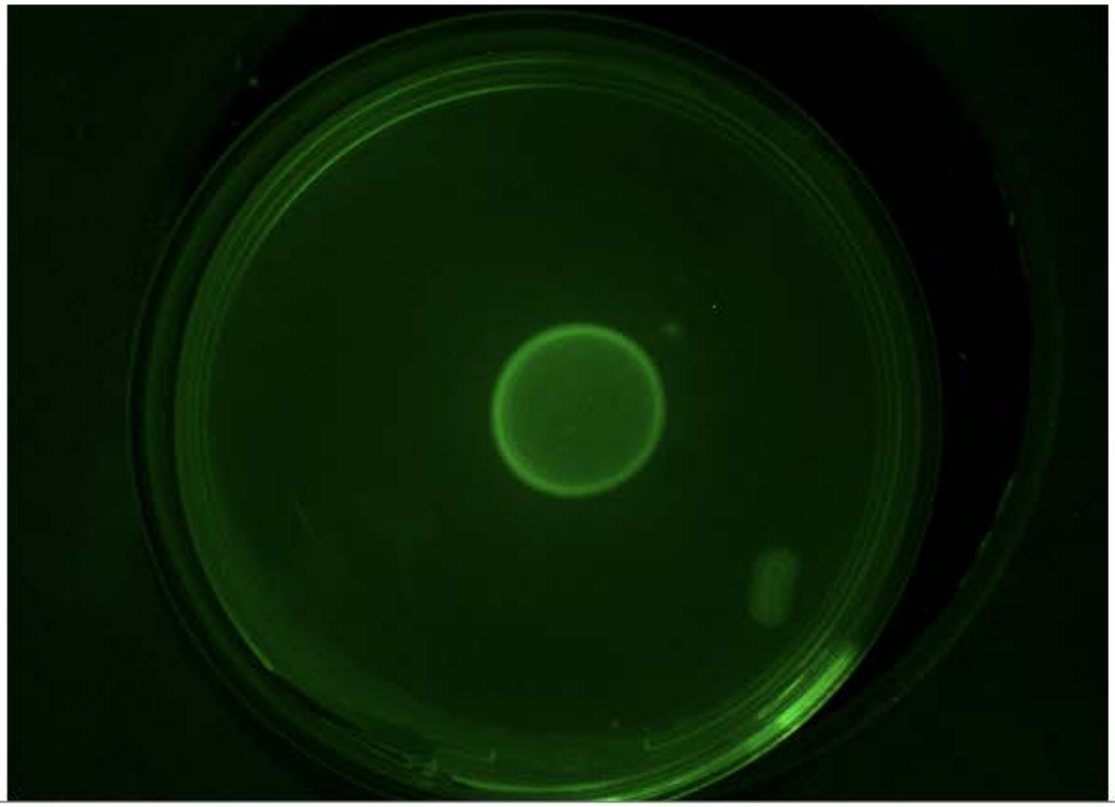
Google form used for movement ratings

Locomotion Assay V1

For this assay, we used GFP E. coli to track worm movement. 3 cm plates were seeded with GFP E. coli, and individual worms were placed near the edge of the plate. The resulting patterns are from the E. coli-coated worm moving around. Please rate the amount of movement you think you see on the plate -- A rating of 1 would indicate very little movement, and 5 indicates a lot of movement. Below are a couple examples to get you started. Try not to rate the brightness of the E. coli, but how much E. coli is spread over the plate. Each rating scale corresponds to the picture above it.

The scores for each plate will be averaged and used in my thesis to compare movement between different treatments of worms. Thanks for participating!

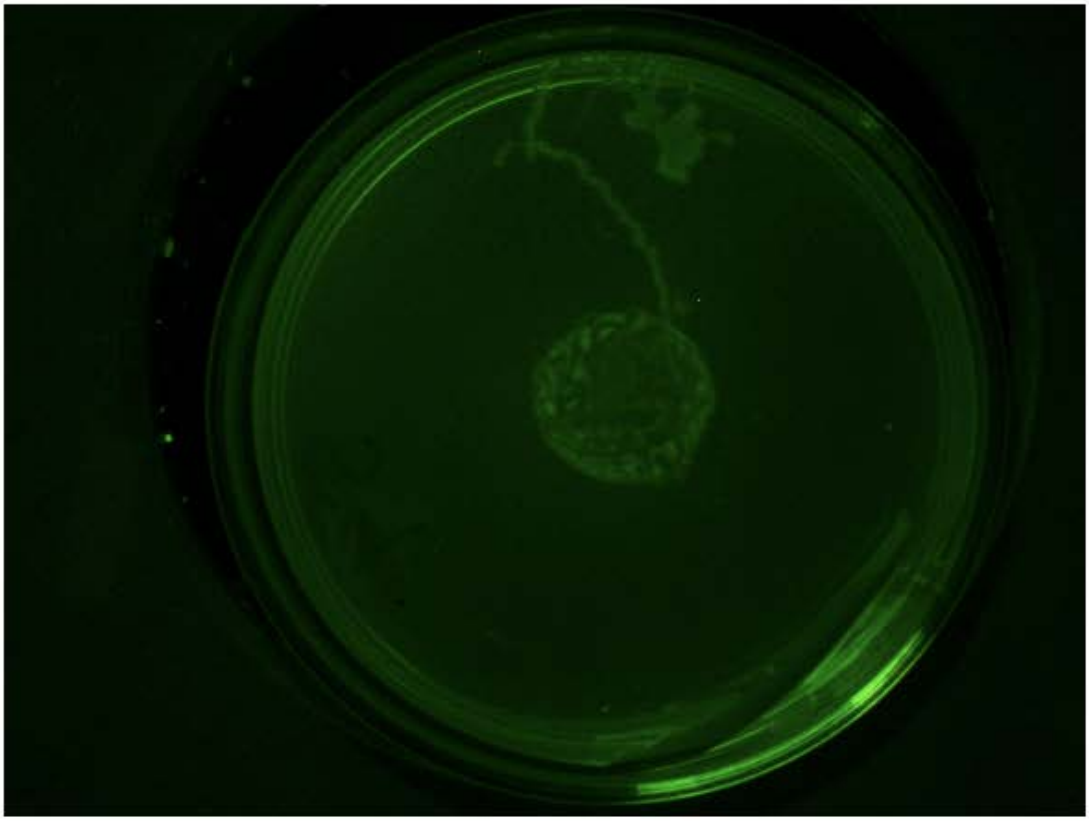
This is considered a 1 because the worm never moved from where it was placed on the plate



Google form continued

Movement Ratings

59



Rate the amount of movement from 1-5.

1 2 3 4 5

Raw Data from Locomotion Assay

	Male	Virgin	Mated	Super-mated
Average Score (15 scores each) per plate	3.8	3.3	2	2.3
	3.6	3.7	2.8	2.5
	4.1	4. 9	2.1	2.2
	4.7	2.8	3.4	2.6
	3.2	2.2	2.1	2.1
	4.9	2.9	2.6	2.5
	4.2	4.0	2.5	2.8
	4.5	4.1	4.	3.9
	4.7	1.7	2.6	2.1
	4.9	3.2	1.9	1.1
	4.56	4.3	1.75	2.6
	3.2	3.2	2.2	1.3
	4.2	3.7	2.5	1.1
	4.7	4.0	2.5	1.3
	3.8	2.8	1	2.5
Average score per treatment	4.2	3.4	2.4	2.2
Avg St. Deviation	0.53	0.56	0.51	0.59

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