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Genetic Determinants of Increased Outcrossing in Caenorhabditis elegans Following Dauer Diapause

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Abstract

Mating strategies, whether sexual or asexual, confer unique costs and benefits to populations and species that facilitate evolutionary processes. In wild isolates of *Caenorhabditis elegans*, mating strategies are dependent on developmental history. Outcrossing levels significantly increase when both hermaphrodites and males have transiently passed through the stress-resistant dauer diapause stage (postdauers). However, the molecular mechanisms of how life history can alter mating strategies have not been systematically explored. Mating behaviors of *C. elegans* can be attributed, in part, to sex-specific responses to ascaroside pheromone components. For example, high concentrations of the pheromone ascr#3 results in a strong avoidance response in hermaphrodites, but a slight attraction in males. We have demonstrated previously that postdauer hermaphrodites exhibit a decreased avoidance of ascr#3, which is the result of the downregulation of the *osm-9* TRPV channel gene in postdauer ADL neurons. Thus, we hypothesized that sex-specific altered detection and/or response to pheromone components in postdauer animals could contribute to their increased outcrossing phenotype.

To test this hypothesis, we conducted mating assays using N2 Bristol and strains carrying mutations in *daf-3*/SMAD and *mut-16*/Mutator genes, which are required for the downregulation of *osm-9* in postdauer hermaphrodite ADL neurons. First, we determined that the outcrossing level of N2 Bristol is primarily correlated with the developmental history of males, with a smaller effect due to hermaphrodites. Interestingly, postdauer males did not downregulate *osm-9* in their ADL neurons, but instead exhibited an increased ability to detect mates via pheromone compared to control males. Additionally, *daf-3* mutants exhibited loss of the increased outcrossing

phenotype in postdauers, illustrating the requirement of the TGF-β pathway in both males and hermaphrodites for this phenotype. Furthermore, the *mut-16* strain exhibited negligible outcrossing, and attempts to rescue the outcrossing phenotype resulted in sterility due to gross morphological defects in the germline. Together, our results suggest a model whereby mating strategy is under combinatorial control of TGF-β and RNAi pathways.

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by

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Chapter 1: Introduction

1.1 Models and theories of reproductive strategies

The theoretical cost of sex and its disadvantages as a mode of reproduction are far higher than that of asexual reproduction (Otto 2009). Yet most species utilize, at least in part, sexual reproduction. What then creates this paradox and makes sex more favorable than self-fertilization and asexual reproduction? The answer to this depends largely on the interplay between the benefits and the costs of different reproductive modes, in addition to the organism's environment.

The main cost of sexual reproduction is the proposed twofold cost of males towards propagation efficiency which has been described since the 1970s, and recently supported by experimental evidence in Gibson *et al.* (Maynard Smith 1971, 1978; Butlin 2002; Gibson *et al.* 2017). The twofold cost refers to the idea that, all else being equal, anisogamous species (species that produce two types of gametes) produce 50% sons that have little contribution to the offspring and 50% daughters that will invest a significant amount of resources in their progeny. In contrast, parthenogenetic species (asexually-reproducing species) produce 100% daughter which in turn produce 100% daughters, effectively doubling their propagation efficiency (Fig. 1). This was shown to be true experimentally using snail populations containing both

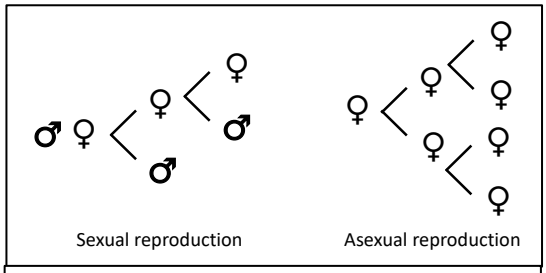


Figure 1. The two-fold cost of sex. Under an all-else-equal assumption, asexually reproducing organisms have twice as many offspring as those that sexually reproduce. Adapted from Gibson *et al.*, 2017.

sexual and asexual females; using mesocosms, the frequency of asexual snails was significantly higher in the progeny compared to the parental generation due to the proposed low cost of asexual reproduction compared to sexual reproduction. Some early theories attributed this twofold cost to genome dilution since each parent is passing on half of its genome to its offspring during sexual reproduction; this theory was quickly rejected since selection does not act on individuals, but on genes (Williams 1975; Treisman and Dawkins 1976; Lehtonen *et al.* 2012). For populations that are well adapted to their environment, sexual reproduction can break up beneficial allelic combinations and linkage groups, leading to decreased fitness (Otto 2009). This, however, has been shown to be a short-term disadvantage allowing for the long-term advantage of creating more genetic diversity and variability (Becks and Agrawal 2012).

In certain cases, sexual reproduction can decrease variation in a given population. When homozygotes are more fit and adaptive than their heterozygous siblings, selection will act to increase the proportion of homozygotes and decrease the proportion of heterozygotes in the population. Sexual reproduction will work against that (due to Hardy-Weinberg principles promoting a higher heterozygote to homozygote proportion of either

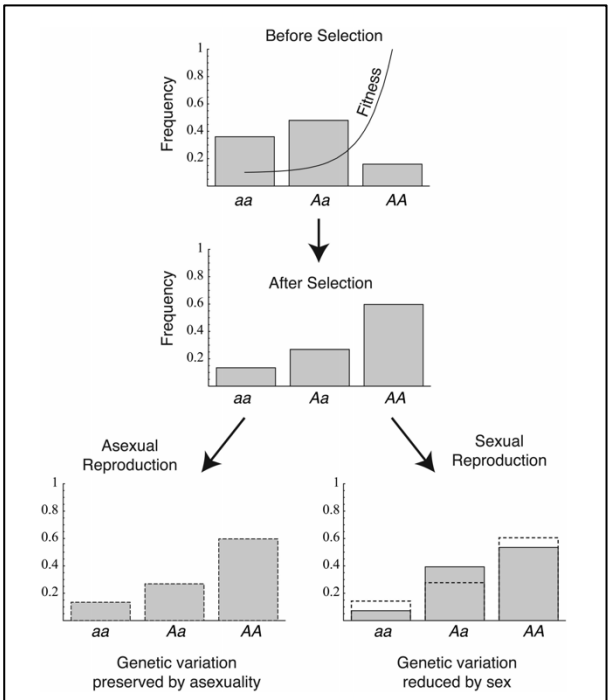


Figure 2. Effect of reproduction mode on variation. Excess homozygosity established by selection can be reduced by sexual reproduction in accordance with Hardy- Weinberg principles. Figure from Otto, 2009.

genotype) and in this case decrease the genetic variation that was established by selection by increasing the frequency of heterozygotes (Fig. 2) (Otto 2009). Finally, sexual reproduction introduces the risk of sexually transmitted diseases, requires mate location which deprives individuals of reproductive assurance, and increases the risk of predation (Meirmans *et al.* 2012; Lehtonen *et al.* 2012).

To offset these costs, sexual reproduction must provide benefits that allowed it to become the dominant mode of reproduction for over 99.9% of eukaryotes. The main advantage that allowed sex to evolve and persist as the major mode of reproduction is recombination. Meiotic recombination allows for the generation of allelic diversity, the purge of deleterious mutations in finite populations through sexual selection, the fixation of beneficial genetic variants, and the increase in phenotypic diversity and the rate of adaptation (Weismann 1889; Burt 2000; Whitlock 2000; Agrawal 2001; Siller 2001; Paland and Lynch 2006; Whitlock and Agrawal 2009). It has been shown experimentally that outcrossing is favored over self-fertilization in novel environments and under a higher mutational rate (Morran *et al.* 2009b). Similarly, facultative sexual reproduction in rotifers has been shown to better support adaptation than asexual reproduction (Becks and Agrawal 2012). Adverse environments favor sexual reproduction since it allows for the production of novel allelic combinations that will potentially increase fitness in that population under the new environmental conditions (Otto 2009). Sexually reproducing organisms use this mode of reproduction to create genetic diversity in their habitats driven by intraspecific competition, allowing them to expand their niches and decrease competition over resources (Bell 1982; Trivers 1983). Additionally, the Red Queen hypothesis (the idea that two species, usually in the context of hosts and parasites, are constantly coevolving in response to each other) supports a

sexually reproductive model since it allows for the genetic diversity and the adaptation rate that promotes the evolutionary arms race, decreasing interspecific competition (Bell 1982; Trivers 1983; Otto 2009; Lively 2010).

While asexual reproduction does provide certain benefits such as reproductive assurance and an increase in propagation efficiency (Baker 1955; Otto 2009), the disadvantages of asexual reproduction also play a role in allowing sexual reproduction to evolve as the main reproductive strategy. Muller's ratchet is a concept that refers to one of the main costs of asexual reproduction: the rapid accumulation of deleterious mutations in asexually reproducing organisms (Muller 1964; Paland and Lynch 2006). This is accompanied by decreased genetic diversity, reduced adaptation rate, and increased inbreeding depression (Charlesworth and Charlesworth 1987; Charlesworth *et al.* 1993; Butlin 2002). Asexually reproducing species tend to be found in new, extreme habitats due to their ability to more quickly invade those habitats and utilize resources, taking advantage of low intraspecific competition (Bell 1982; Trivers 1983).

1.2 Self-fertilization vs. outcrossing

While the benefits and costs of the two main reproduction modes, sexual and asexual reproduction, were discussed in the previous section, different types of sexual reproduction exist. Although both are considered forms of sexual reproduction, outcrossing and self-fertilization confer different benefits on individuals and populations but are associated with particular costs. Outcrossing refers to sexual reproduction between two individuals of different sexes, while self-fertilization, or "selfing", occurs within the same individual. Considering the cost and benefits of sexual

and asexual reproduction, mixed mating systems represent a very attractive alternative to having to commit to one mode and suffering its costs.

Evolutionary models have theorized how outcrossing might have evolved, referring to the benefit of reduced inbreeding depression in selfing populations. Using the model *Caenorhabditis elegans,* work by Asher Cutter has shown experimentally that increased mutational load using a genetic background prone to mutation accumulation is associated with an increase in outcrossing (Cutter 2005). However, this explanation is not solely responsible for the rise of obligate outcrossing, since self-fertilization persisted in populations exposed to high mutation loads, even in a trioecious population (both hermaphrodites, males, and females present in the population). Similar conclusions were drawn from an experimental study that used mutagens instead of an allelic variation and extended the study to different wild isolates of the same species (Manoel *et al.* 2007).

Due to the beneficial nature of facultative outcrossing, multiple models have failed to show obligate sex invading and replacing facultative sex when it is an option in a certain population (Kokko 2020). Outcrossing species have a lower level of linkage disequilibrium compared to selfing species, making it less likely for deleterious alleles to "hitch-hike" in strong linkage groups (Flint-Garcia *et al.* 2003; Glémin *et al.* 2006, 2019). Moreover, outcrossing species have higher genetic diversity and sequence polymorphism compared to selfing species due to the higher levels of homozygosity in selfers (Hamrigk and Godt 1996; Glémin *et al.* 2006, 2019). Additionally, facultative outcrossing offers one of the greatest advantages of sexual reproduction without suffering the twofold cost of producing males: increased adaptation rate (Green and Noakes 1995; Kokko 2020).

In addition to the benefits mentioned above, facultative outcrossing avoids the costs of self-fertilization; mainly the reduced rate of effective recombination and increased rate of mutation accumulation (Nordborg 2000; Baer *et al.* 2010; Glémin *et al.* 2019). Conversely, outcrossing has costs that are mitigated by self-fertilization such as the concept of Haldane's sieve. This phenomenon refers to new beneficial alleles being less likely to be fixed in outcrossing populations since they are not visible to selection due to the high heterozygosity of outcrossing populations (Haldane 1937; Glémin *et al.* 2019). Sexual selection can promote the evolution of costly traits that are required or highly advantageous for sexual reproduction, such as the copulatory plugs that are present following copulation in certain gonochoristic *Caenorhabditis* species but are absent in androdioecious species (Hodgkin and Doniach 1997; Palopoli *et al.* 2008; Cutter 2008). Sexual conflict is another cost of sex that refers to the evolution of alleles that are beneficial to one sex but deleterious to the other (Mank 2017). The literature is rich in examples of how detrimental sexual reproduction can be, from decreasing an organism's life span to killing the mate during or post-copulation (Gems and Riddle 1996; Gendron *et al.* 2014; Maures *et al.* 2014; Shi and Murphy 2014; Schneider 2014; Huffard and Bartick 2015; Barry 2015; Shi *et al.* 2017). Thus, mixed mating systems offer the opportunity to harvest the benefits of both outcrossing and self-fertilization by promoting either mode depending on developmental or environmental conditions.

1.3 Environmental influence on mating strategies

Changes in reproductive strategies mediated by phenotypic plasticity are driven by environmental conditions. Phenotypic plasticity is a developmental process that

refers to the capacity of a single genotype to produce multiple phenotypes depending on environmental conditions (Pigliucci *et al.* 2006). It can be associated with beneficial outcomes, such as driving adaptation, or detrimental outcomes, such as cancer cell heterogeneity (Gupta *et al.* 2019). There is a multitude of organisms that can express such phenotypic plasticity ranging from crustaceans and nematodes to plants and humans. These phenotypic plasticity-dependent changes provide a unique value to populations since they result in relatively quick, generational changes to reproductive strategies.

The literature has a plethora of examples of species that can alter their reproductive strategies in response to environmental changes. Daphnids can switch from asexual to sexual reproduction once male sex determination is initiated in response to environmental stresses such as a decrease in temperatures and starvation (LeBlanc and Medlock 2015). Additionally, plants use genetic variation and environmental cues to modulate their outcrossing rates (Holtsford and Ellstrand 1992). Certain plants increase the rate of self-fertilization with increased temperatures (Johnson 1971; Campbell and Linskens 1984; Levin 2012). Herkogamy, the separation between the anthers and the stigma, is dependent on environmental factors such as temperature, and can mediate this change in self-compatibility since decreased herkogamy promotes increased self-fertilization (Rick *et al.* 1977; Brock and Weinig 2007; Levin 2012; Luo and Widmer 2013). Other factors such as the presence or absence of pollinators can have an effect on herkogamy levels (Bodbyl Roels and Kelly 2011). Cleistogamous species, plant species with flowers that are obligate selfers (cleistogamous) or flowers that can promote cross-fertilization (chasmogamous) on the same plants, also show an increase in the proportion of cleistogamous flowers under

adverse conditions such as low nutrients, light, and water availability (Oakley *et al.* 2007).

Nematode species such as *Caenorhabditis* also exhibit phenotypic plasticity in their mating strategies. Certain *Rhabditis* species are trioecious and can reproduce via mating between males and females only when they bypass a diapause stage called dauer. When they experience dauer, they develop as protandrous hermaphrodites (Félix 2004; Chaudhuri *et al.* 2011). While nematodes of the *C. elegans* species are primarily hermaphrodites, males can be found at extremely low frequencies in the population due to rare nondisjunction events of the X-chromosome (Nigon 1949; Hodgkin *et al.* 1979). These gonochoristic males allow for hermaphrodites to switch from self-fertilization to outcrossing in the same animal. It has been shown that the frequency of outcrossing increases when animals have experienced adverse conditions early in life such as starvation (Morran *et al.* 2009a). The molecular mechanism underlying these stress-dependent changes in mating strategies is not well understood.

1.4 *Caenorhabditis elegans* as a model organism

Ever since it was developed by Sydney Brenner in the 1960s as a model organism for genetic research, *C. elegans* has been an indispensable genetic tool. It has aided in the discovery of many important biological processes and phenomena such as RNA interference and the mechanism of programmed cell death (Sulston *et al.* 1983; Fire *et al.* 1998). It was the first organism to have its entire cell lineage mapped and its genome sequenced (Sulston and Horvitz 1977; Sulston *et al.* 1983; The *C. elegans* Sequencing Consortium 1998). In addition to its short life span and ease of cultivation in a laboratory setting, the hermaphroditic nature of *C. elegans* also allows for the use of

large, essentially clonal populations in experiments. What makes this model organism particularly attractive to study the plasticity of reproductive strategies in response to adverse environments is its capability of developmental arrest. Under favorable conditions, *C. elegans* will develop through four larval stages before becoming a reproductive adult. However, if they experience harsh conditions such as starvation, high temperature, or crowding, young larva will enter the stress-resistant dispersal state called dauer (Cassada and Russell 1975; Golden and Riddle 1982, 1984). They can remain as dauers for several months and, once conditions improve, they resume their development and become reproductive postdauer adults (Cassada and Russell 1975; Golden and Riddle 1982). Interestingly, it has been shown that passage through dauer alters reproductive outcomes, gene expression profiles, and behaviors such as chemotaxis and mating behavior (Morran *et al.* 2009a; Hall *et al.* 2010; Sims *et al.* 2016; Vidal *et al.* 2018; Ow *et al.* 2018; Bhattacharya *et al.* 2019).

C. elegans is capable of using environmental cues throughout their developmental history, such as food availability during early larval stages, to influence their mating strategies in order to allow them to switch between self-fertilization and outcrossing. While *C. elegans* populations are composed of primarily self-fertilizing hermaphrodites, it has been shown that postdauer animals increase facultative outcrossing with males when compared to continuously developed animals of different wild isolates (Morran *et al.* 2009a). Thus, normally selfing populations increase outcrossing frequencies under unfavorable conditions. While these differential outcrossing phenotypes have been well described in different wild isolates, it has not been yet reported for the commonly used *C. elegans* lab strain, N2 Bristol. Additionally,

the molecular mechanism underlying the phenotypic plasticity of reproductive strategies is still not well understood.

1.5 Mating in *C. elegans*

Mating is one of the most complex behaviors that *C. elegans* displays; it requires males to locate mates based on mating signals secreted by the hermaphrodites. Early research efforts found that hermaphrodites produce a "mate-finding" pheromone cue that is detected by males, allowing them to switch from foraging behavior to mate-locating behavior (Lipton et al., 2004; Simon and Sternberg, 2002; see section 1.6). Notably, this cue is independent of vulval tissue and was thought to depend on chemosensory circuits (Simon and Sternberg 2002). Males then perform a stereotyped process that is concluded by sperm transfer into the hermaphrodites if the mating is successful; otherwise, the hermaphrodite can escape the male by "sprinting" away. Mating in *C. elegans* begins once the male locates the hermaphrodite. The male responds to the mechanosensory stimulus and scans the body of the hermaphrodite through backward locomotion utilizing the sensory rays in its tail, turning around the hermaphrodite's body multiple times until the vulva is located. Then, the male can insert its spicules and ejaculate, concluding this intricate behavior (Barr and Garcia 2006). The sensory structures that are required for mating such as the sensory rays, the spicules, the hook, and the postcloacal sensilla have been determined for each step of this stereotyped behavior (Liu and Sternberg 1995).

The frequency and success of mating are influenced by many factors such as the abundance of self-sperm, the time since the last mating event, the hermaphrodite's ability to escape males, and developmental history. There is a striking, naturally

occurring variation in mating frequency between different wild isolates of *C. elegans*. For example, N2 Bristol strain is less successful at mating than the Hawaiian strain CB4856. This difference is due to the fact that N2 hermaphrodites actively avoid males, whereas Hawaiian hermaphrodites do not (Bahrami and Zhang 2013). It has also been reported that *C. elegans* has an increase in facultative outcrossing that is correlated with depletion of hermaphrodite self-sperm and due to a decrease in mating resistance (Kleemann and Basolo 2007). This age-independent decrease in resistance is mainly due to three factors: a decrease in hermaphrodite "sprinting" away from mates, a reduction in mating time, and a decrease in ejecting male sperm following copulation (Kleemann and Basolo 2007). Older, sperm-depleted hermaphrodites produce a signal that is attractive to males, and this depends on the sperm-sensing pathway by the soma (Morsci *et al.* 2011). This cue, which has been determined to not be a short-chain ascaroside, is suppressed by mating (Morsci *et al.* 2011).

It has also been shown that hermaphrodites actively avoid mating under certain circumstances by utilizing either or both chemosensory and mechanosensory inputs (Bahrami and Zhang 2013). Mechanosensory input is required for hermaphrodites to exhibit decreased mating frequency through mating resistance following the perception of males. This is supported by results showing a significantly increased mating frequency of *mec-3* and *mec-4* (both are required for the proper cell-fate determination of the mechanosensory neurons) mutants. This mating avoidance by hermaphrodites also depends on chemosensory neurons expressing cGMP channels (*tax-2/tax-4*) (Bahrami and Zhang 2013).

Mating has detrimental effects on both hermaphrodites and males. *C. elegans* has reduced life span and body shrinkage following mating (Gems and Riddle 1996; Shi and

Murphy 2014; Shi *et al.* 2017). This effect is independent of gamete production and sperm transfer as the reduced lifespan is observed in sterile individuals that fail to produce gametes (Gems and Riddle 1996). The detrimental effect of mating with males is summarized in hermaphrodite shrinking, a decrease in lifespan, and death. By using males that lacked both seminal fluid and sperm, Shi *et al.* showed that the lifespan decrease and body shrinking is independent of copulation but instead depends on either sperm or seminal fluid (Shi and Murphy 2014).

While mating is detrimental to hermaphrodites, it also has a negative effect on males. Similar to the effects in hermaphrodites, mating induces shrinking and death in males (Shi *et al.* 2017). There is a significant decrease in lifespan, up to 35%, when comparing solitary males to males exposed to hermaphrodites (Shi *et al.* 2017). The decrease in lifespan directly correlates with the length of mating period; the longer males are exposed and mated with hermaphrodites, the shorter their lifespan is (Shi *et al.* 2017). Moreover, male density-dependent death has been observed in androdioecious *Caenorhabditis* species but not in gonochoristic ones. This toxic nature of male pheromones induces adverse effects in males, and it is less effective on hermaphrodites. However, the toxicity significantly increases in masculinized hermaphrodites (Shi *et al.* 2017). This male-pheromone toxicity is independent of the decrease of lifespan following mating, and it acts through a distinct mechanism. Additionally, conditioning the growth media with male pheromone throughout development significantly decreases lifespan in addition to a decrease in male fertility for up to two generations (Shi *et al.* 2017). Decreased lifespan has been observed following exposure to male-conditioned media (Maures *et al.* 2014). This effect requires the insulin-like peptide INS-11 in the sensory neurons in hermaphrodites (Maures *et al.*

2014). Although these examples reflect the immediate cost of sexual reproduction in *C. elegans*, the evolutionary benefit of decreased lifespan in hermaphrodites is an increase in male mating success through accelerating the developmental rate of hermaphrodites, thus reaching reproductive age early (Ludewig *et al.* 2019).

1.6 Pheromone detection in *C. elegans*

C. elegans detects an array of social cues through pheromones that trigger a variety of behaviors and developmental decisions such as dauer entry, mating, and aggregation (Golden and Riddle 1982; MacOsko *et al.* 2009). Interestingly, mating systems influence pheromones' production and the responses they illicit. While females from the gonochoristic species *Caenorhabditis remanei* and *Caenorhabditis brenneri* secrete a potent "sex pheromone" that attracts males, the production of this pheromone is suppressed under many conditions in the closely related androdioecious species *C. elegans* (Chasnov *et al.* 2007; Leighton *et al.* 2014). *C. elegans* hermaphrodites produce this volatile pheromone only when they deplete their sperm or in the absence of recent mating events such that old hermaphrodites and young, sperm-depleted hermaphrodites are more attractive to males (Leighton *et al.* 2014).

The pheromones produced by hermaphrodites consist primarily of ascaroside molecules (Butcher *et al.* 2007). Ascarosides (ascr), first identified in the roundworm family *Ascaridia,* refers to derivatives of dideoxysugar ascarylose with a carbon chain of variable lengths (Jeong *et al.* 2005; Butcher *et al.* 2007; Ludewig and Schroeder 2013). When ascarosides are named, the number of carbons in the side chain is identified in the name; the ascaroside C9 (ascr#3) is an ascaroside containing 9 carbons in its side chain. The other naming system refers to the order of which these ascarosides were

identifies; ascr#3 was the third dauer pheromone that was identified as a potent dauer-inducing pheromone (Butcher *et al.* 2007). Hermaphrodites produce a blend of pheromones including ascr#2, ascr#3, and ascr#4 at low concentrations that act synergistically to make up the "mating signal" described previously, distinct from the "sex pheromones" produced by other species described above (Srinivasan *et al.* 2008).

Males and hermaphrodites have distinct neuronal circuitry that allows them to exhibit sex-specific responses to pheromones (Barr *et al.* 2018). Of the total number of neurons in *C. elegans,* 294 are shared between the two sexes, while 8 neurons are hermaphrodite-specific and 91 are male-specific (Barr *et al.* 2018). While there is a high proportion of shared neurons, some are functionally different between the two sexes (Jang *et al.* 2012). Hermaphrodites first sense ascr#3 via the ADL and ASK neurons, ADL promoting avoidance and ASK promoting attraction (Fig. 3) (Srinivasan *et al.* 2008; Jang *et al.* 2012). The antagonism between the two neurons, mediated by the RMG inter/motor neuron gap junctions, ultimately determines the behavior performed. While the shared ADF neurons are capable of detecting ascr#3, the sexual state of these neurons, as determined by the master regulator *tra*-1 transcription factor, plays a role in their functionality, such that hermaphrodite ADF neurons do not respond to ascr#3 (Fig. 3) (Wolff and Zarkower 2008; MacOsko *et al.* 2009; Fagan *et al.* 2018). Hermaphrodites avoid ascr#3 via the ADL neurons and downstream chemical synapses (Jang *et al.* 2012). This avoidance in hermaphrodites is significantly decreased when the animals experience the stress-induced dauer diapause (Hall *et al.* 2010; Sims *et al.* 2016).

The hub-and-spoke RMG circuit, where the central "hub" interneuron (RMG) coordinates the inputs of multiple "spoke" neurons in the circuit (ASK and ADL), is

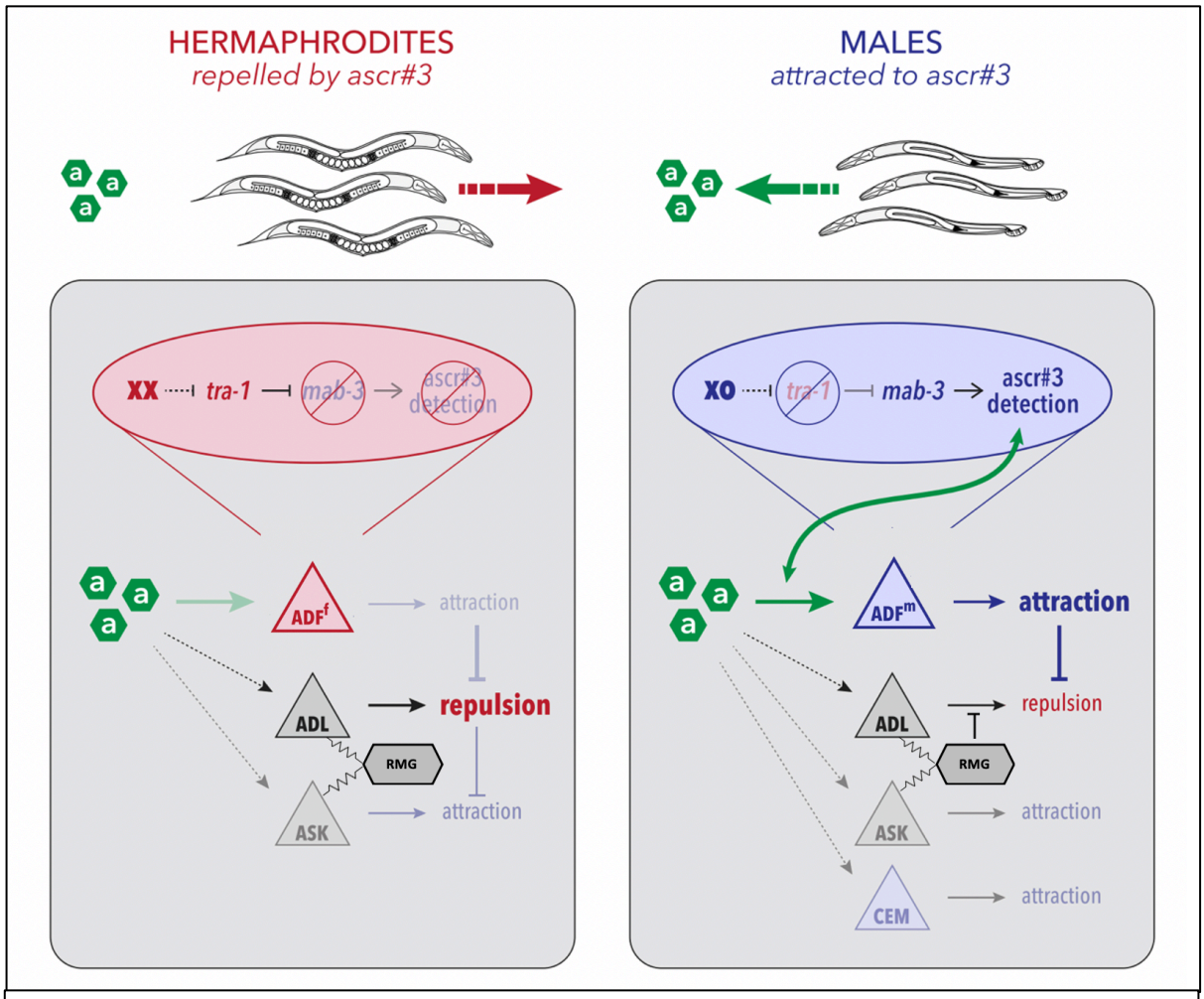


Figure 3. Neuronal circuitry of the response to ascr#3. Hermaphrodites utilize ADL neurons to promote repulsion, while males rely on ASK, CEM, and masculinized ADF neurons to generate attraction behavior in response to ascr#3. RMG inter/ motor neuron is connected to ADL and ASK through electrical synapses, mediating the "push-pull" dynamic between the two chemosensory neurons, and inhibiting repulsion in males. Figure adapted from Jang *et al.,* 2012 and Fagan *et al.,* 2018.

modified in males, suppressing the avoidance behavior, and instead promoting attraction (Jang *et al.* 2012). The RMG inter/ motor neuron in males antagonizes the ADL neurons, allowing for the attraction response via the ASK neurons (Fig. 3) (Jang *et al.* 2012). The sex-specific CEM neurons also contribute to the attraction response to ascr#3 in males (Fig. 3) (Srinivasan *et al.* 2008; Jang *et al.* 2012). Finally, the

masculinized ADF neurons further promote attraction to ascr#3 in males, by responding to ascr#3 in a sex-specific manner (Fig. 3) (Fagan *et al.* 2018).

In addition to the neuronal circuitry, the sexually dimorphic response to ascr#3 depends on the expression of the transient receptor potential vanilloid (TRPV) channel OSM-9 in certain sensory neurons (White *et al.* 2007). OSM-9, an ortholog of the human TRPV5, encodes for a capsaicin receptor-like TRPV channel that is required for olfactory behaviors and osmotic avoidance (Colbert and Bargmann 1995; Colbert *et al.* 1997). In hermaphrodites, the expression of *osm-9* has been shown to depend on developmental history; hermaphrodites that experience dauer downregulate *osm-9* in the ADL neurons (Sims *et al.* 2016). We hypothesize that this change in pheromone detection and response can potentially contribute to the previously reported changes in outcrossing following stress (Morran *et al.* 2009a).

The impact of *osm-9* on mating frequency has been previously described in hermaphrodites (Bahrami and Zhang 2013). The researchers investigated different factors and pathways that lead N2 Bristol to have a decreased outcrossing rate compared to the wild isolate CB4856. *osm-9* mutants were tested to examine the levels of outcrossing compared to the wild-type N2 Bristol strain. It was found that the outcrossing rate was not significantly different in the absence of functional *osm-9,* leading to the conclusion that chemosensory neurons that express *osm*-9 are not required to generate the low mating frequency observed in N2, compared to other wild isolates (Bahrami and Zhang 2013). However, the role that the differential expression of *osm-9* in postdauers play in regulating outcrossing frequencies has not been explored.

1.7 *osm-9* regulation and plasticity of pheromone detection

As mentioned in the previous section, pheromone detection and response are plastic attributes of many species, including *C. elegans*. It has been shown previously that the downregulation of *osm-9* TRPV in ADL neurons leads to a decrease in ascr#3 avoidance in hermaphrodites following dauer diapause (Sims *et al.* 2016). OSM-9 mediates sensory transduction when localized to the worm's sensory cilia with OCR-2, another TRPV channel (Tobin *et al.* 2002). When localized to the cell body, however, it mediates sensory adaptation (Tobin *et al.* 2002). It is also required for mechanosensation in a sex-specific manner: it mediates mechanical signal transduction in the male ray neurons (Zhang *et al.* 2018).

The downregulation of *osm*-9 gene in postdauer hermaphrodites, specifically in the ADL neurons, is dependent on the RNAi, TGF- β, and chromatin remodeling pathways (Fig. 4) (Sims *et al.* 2016). When food is readily available for hermaphrodites,

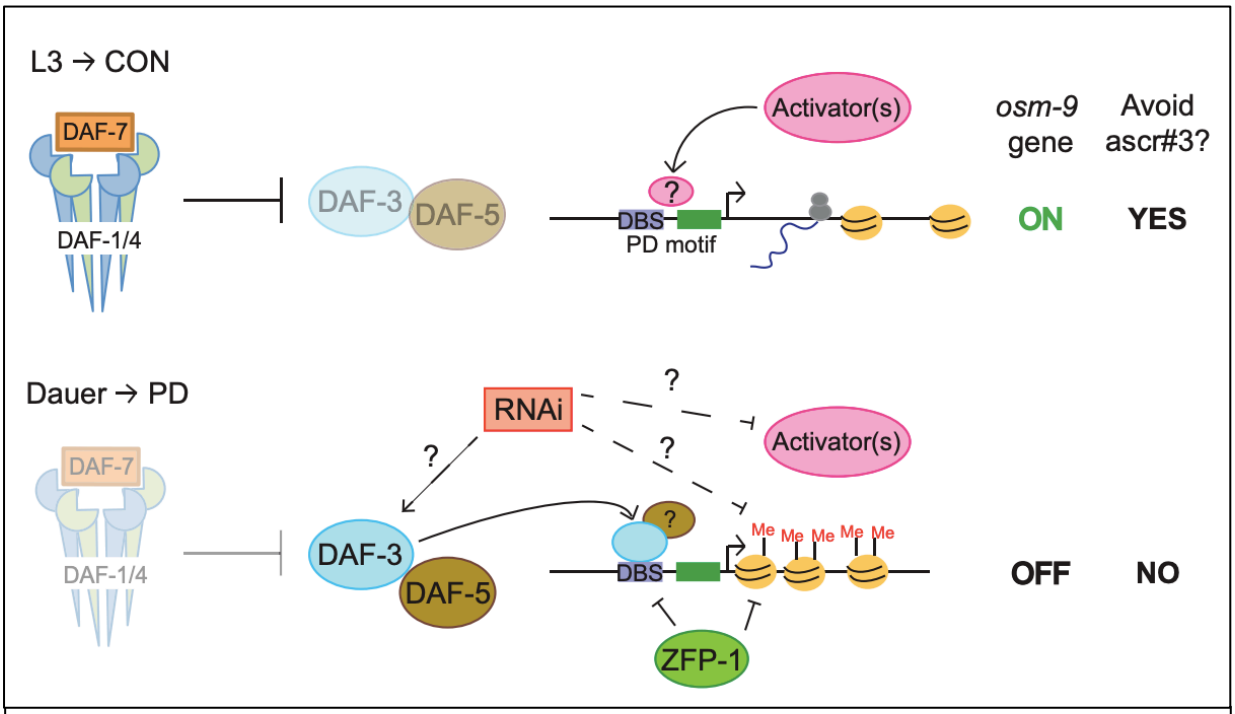


Figure 4. The mechanism of *osm-9* downregulation in hermaphrodites. The proposed mechanism of *osm-9* downregulation involves DAF-3, ZFP-1, MUT-16, and NRDE-3 activity in order to regulate its expression. Figure from Sims *et al.,* 2016.

daf-7 TGF- β is expressed and its product is able to bind to type I and II receptors, DAF-1 and DAF-4. Downstream, this prevents the DAF-3 SMAD from binding to the *osm-9* promoter, instead allowing activators to bind and promote *osm-9* expression. This permits ascr#3 avoidance in hermaphrodites. However, when hermaphrodites experience dauer diapause, they no longer express DAF-7 TGF- β. Thus DAF-3 SMAD is now able to bind and silence *osm-9*, rendering hermaphrodites unable to avoid ascr#3. While the mechanism for *osm-9* regulation has been resolved in hermaphrodites, how *osm-9* is regulated in postdauer males has not yet been investigated.

Mutations in *mut-16* and *zfp-1,* which are genes that have functions in RNAi and chromatin remodeling pathways, respectively, also fail to suppress *osm-9* expression in ADL neurons in postdauers (Sims *et al.* 2016). Additionally, a null mutation in the argonaute *nrde-3*, a member of the endogenous siRNA pathway, also leads to derepression of *osm-9* in postdauer hermaphrodites (Sims *et al.* 2016). Interestingly, these models are mechanistically connected, as DAF-3 is not enriched at the *osm-9* promoter in postdauer adults in *mut-16* and *zfp-1* mutant strains. Thus, a model was constructed where *osm-9* is under combinatorial regulation of the pathways mentioned above, and a defect in any one of these pathways leads to misregulation of *osm-9* in response to environmental stress (Sims *et al.* 2016).

1.8 Hypothesis

Since pheromones are key to mating behaviors in *C. elegans*, we hypothesize that the change in ascr#3 pheromone detection in postdauer hermaphrodites could potentially underlie changes in reproductive strategies following passage through dauer. When hermaphrodites do not actively avoid high concentrations of pheromones, this

behavior potentially increases opportunities of males successfully locating mates. Thus, we hypothesize that changes in pheromone detection or the response to pheromones by postdauer animals are contributing to the increase in facultative outcrossing frequency.

In chapter 2, we investigate the molecular mechanisms underlying the increased outcrossing phenotype, and whether the commonly used N2 Bristol exhibits increased outcrossing following passage through stress. One of our findings in that chapter illustrated severe fecundity defects in *mut-16* rescue strains. In chapter 3, we investigate the germline defects that are induced by *mut-16* rescue extrachromosomal arrays and whether these defects are heritable transgenerationally. The results in chapters 2 and 3 are being prepared for publication. Overall, this project examined how epigenetic changes in gene expression patterns influence mating behavior and identified proteins and pathways that are required for the phenotypic plasticity of reproductive strategies. This provides another example that highlights the benefits of mixed mating strategies and demonstrates how environmental conditions can drive changes in mating systems, ultimately driving adaptation.

Chapter 2: Male-dependent increase of outcrossing following diapause

2.1 Introduction

The previous chapter discussed the importance of phenotypic plasticity in reproductive systems as a means for adaptation and maintaining or increasing genetic diversity in a given population. The molecular mechanism underlying this change in mating systems, however, has not yet been elucidated. *C. elegans* represents an ideal model organism to study such mechanisms, as it exhibits phenotypic plasticity in reproductive strategies in response to stress exposure. Morran *et al.* first described the change in *C. elegans* mating behavior following starvation stress. The goal of the study was to understand the effects of stress on facultative outcrossing and whether stress induces an increase in facultative outcrossing in primarily self-fertilizing populations (Morran *et al.* 2009a). The study first found that the proportion of males in the *C. elegans* JU440 and CB4856, French and Hawaiian wild isolates, respectively, significantly increased following successive dauer exposure (Morran *et al.* 2009a). This result was observed whether the initial populations contained 0% or 10% males (Morran *et al.* 2009a). However, this was not observed in the N2 Bristol strain, thus subsequent analyses focused mainly on the French and Hawaiian isolates. The strains that exhibited an increase in the number of males after dauer exposure in large populations were then tested for increase in outcrossing using mating assays. The increased male proportion following successive dauer exposure is attributed to both a higher male survival compared to hermaphrodites, and an increase in facultative outcrossing rates (Fig. 5) (Morran *et al.* 2009a). Since N2 Bristol did not exhibit a significant increase in males, the levels of outcrossing for this isolate was not measured in Morran *et al.* Here, we

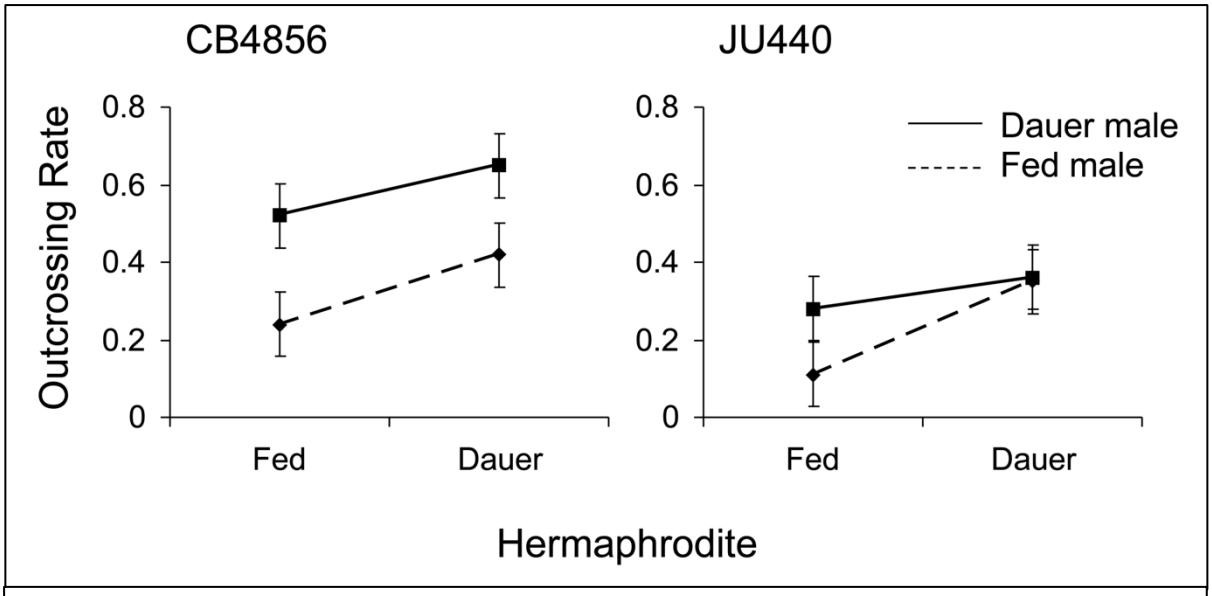


Figure 5. Dauer diapause induces an increase in outcrossing rates. Both CB4856 and JU440 experience an increase in outcrossing rate if either a single parent, or both, experience dauer. The x-axis denotes type of hermaphrodites, while the different lines denote the type of male. Figure from Morran *et al.,* 2008a.

investigate the molecular mechanisms that underlie this increase in facultative outcrossing following dauer diapause.

C. elegans detects social cues though pheromones, triggering a variety of behaviors and developmental decisions such as dauer entry, mating, and aggregation (Golden and Riddle 1982; MacOsko *et al.* 2009). Both males and hermaphrodites produce ascaroside pheromones that elicit sexually dimorphic responses. For example, while hermaphrodites exhibit a strong repulsion by the pheromone ascr#3, males are attracted to that same pheromone component (Simon and Sternberg 2002; Srinivasan *et al.* 2008). In chapter 1, we established the idea of sexual dimorphism in response to mating signals and hypothesized that this is a potential mechanism underlying the increase in facultative outcrossing. Thus, we hypothesize that sexually dimorphic changes in pheromone detection or the response to pheromones by postdauer animals

are contributing to the increase in facultative outcrossing frequency. In this chapter, we first examine whether the commonly used N2 Bristol strain shows the same increased outcrossing phenotype that has been described in other wild isolates after a single dauer exposure using mating assays (Morran *et al.* 2009a). Additionally, we investigate the role that the TGF-β and RNAi pathways play in regulating outcrossing frequencies. Lastly, we investigate the contribution of males and hermaphrodites in determining outcrossing rates following stress.

2.2 Materials and methods

2.2.1 Strain maintenance

The strains used in this study were grown on standard nematode growth media (NGM) plates seeded with *Escherichia coli* OP50 and cultivated at 20°C unless specified otherwise (Appendix 5.1). Due to the temperature-sensitive mortal germline phenotype exhibited by *mut-16(pk710)* and the neuronal rescue strains, they were maintained at 15°C. All strains were maintained at 20°C after dauers were harvested using a standard 1% SDS wash on food-depleted plates that contain a large population of dauers. SDS, sodium dodecyl sulfate, is a chemical commonly used to isolate dauer worms, since they develop a thick cuticle and a buccal plug, making them resistant to SDS (Cassada and Russell 1975; Karp 2016). All the *mut-16* rescues used in this study were made by Dr. Pallavi Bharadwaj (Bharadwaj and Hall 2017).

2.2.2 Mating assays

Mating assays were conducted in the following combinations of continuously developed control (CON) and postdauer (PD) adult males (M) and hermaphrodites (H):

CON-H x CON-M, CON-H x PD-M, PD-H x CON-M, and PD-H x PD-M by allowing six hermaphrodites and six males to mate on a single plate. A few exceptions for these values are listed in Appendix 5.2. The hermaphrodites were picked at the L4 stage (CON) or approximately 24 hours after the SDS wash (PD) on a starved NGM plate. The males were picked at the young adult stage (CON), or approximately 48 hours after the SDS wash (PD) on a starved plate. These starved populations were generated by growing an age-mixed population on 60mm NGM plates and allowing them to grow until food depletion. Approximately 3-5 days following food depletion, the plates are visually examined to check the presence of dauers, then an SDS wash is performed on these starved plates. Since *daf-3(mgDf90)* is a dauer-deficient strain, populations were grown on 100mm NGM plates seeded with concentrated bacteria (20x OP50) at 25°C for a week to induce dauer formation. It is important to note that these dauers are induced via starvation, since temperature induced-dauers form at 27°C (Ailion and Thomas 2000). After the plates were starved and dauers are observed, an SDS wash is used to isolate postdauers. The parents were placed on 60mm NGM plates, allowed to mate for 23 hours, followed by removal of males from the plates. The hermaphrodites were placed on new NGM plates daily until egg-laying ceased, and the number of progeny per plate was determined.

The mating frequency was determined using the proportion of males in the F1 population after mating. Since males are produced through either mating or nondisjunction events of the X-chromosome, the mating frequency can be determined by subtracting the frequency of X-chromosome nondisjunction from the overall observed male frequency (outcrossing rate = $\frac{number\ of\ male\ progeny}{number\ of\ total\ progeny} -$

$X\ Chromosome\ nondisjunction\ frequency$). The X-chromosome nondisjunction frequencies for all the strains used in the mating assays can be found in Appendix 5.3. The number of independent biological replicates and sample sizes, the number of mated hermaphrodites and males combined, can be found in Table 1. To compare the mating assay results of the different conditions, two-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

Cross genotype	Biological replicates (N)	Sample size (n)
N2	≥ 8	408
daf-3(mgDf90)	8	364
N2-H x *daf-3*-M	≥ 3	180
daf-3-H x N2-M	4	192
mut-16(pk710)	3	134
mut-16 pan-neuronal rescue	3	140
mut-16 ASI rescue	3	134
mut-16 ADL rescue	≥ 3	116
mut-16 ADF rescue	5	232

Table 1. Number of biological replicates and sample sizes for mating assays. Biological replicates represent assays set up independently. Sample size represents the total number of hermaphrodites and males combined, that were used to set up the mating assays.

2.2.3 Mate preference and chemotaxis assays

This experiment was conducted at the Portman Laboratory by Dr. Jintao Luo at the University of Rochester. Mate preference assays were conducted in a *him-5* background to provide sufficient males for testing. To test whether males have heightened attraction to hermaphrodites following dauer diapause, CON and PD males were used as the tester worms and immobilized hermaphrodites were used as the targets (Fig. 10A). Each quadrant contained four target worms, with each quadrant alternating between the *unc-54; him-5* and *unc-54; daf-22; him-5* strains. Ten tester worms were placed in the center of the plate and allowed to move freely about the different quadrants. The location of the worms was noted every 30 minutes for up to 120 minutes, and the mate preference index was then calculated as previously described (Fagan *et al.* 2018). A total of 18 trials were performed and 180 animals were tested for this assay. The same method was used for ascr#3 chemotaxis assay, except using 10 mM or 100 mM ascr#3 as the target, ethanol diluted in water as the negative control, and CON and PD males as the testers (Fig. 10E). A total of 10 trials were performed and 100 animals were tested for this assay. To test if hermaphrodites had an increased attraction to mates, the same method was used, with *unc-54; him-5*, CON or PD, males as the targets (Fig. 10C). A total of 16 trials were performed and 160 animals were tested. To compare chemotaxis indices and mate preference indices, unpaired *t*-test was performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

2.2.4 Pheromone avoidance assays

Worms were tested for avoidance behavior in response to 100 mM ascr#3, with 1M glycerol being used as the positive control and M13 buffer as the negative control.

The worms tested were young adult, control or postdauer males and hermaphrodites. The worms were picked onto a 60mm NGM plate without food and allowed to acclimate for about 10 minutes. Then, the drop test acute avoidance response assay is performed using a mouth-pipette and a pulled glass pipette. The first chemical tested is 1M glycerol, used to test approximately 30 animals. M13 is tested second on all the worms, followed by ascr#3. A response is recorded when a forward-crawling worm reversed within two seconds after being exposed to the stimulus. The proportion of worms responding to ascr#3 is normalized by subtracting the proportion of worms responding to the positive control (M13) as previously described (Hilliard *et al.* 2002; Jang *et al.* 2012; Sims *et al.* 2016). The number of biological replicates for ascr#3 avoidance and

Response to ascr#3				
Genotype	**Sex**	**Condition**	**Biological replicates (N)**	**Sample size (n)**
N2	Hermaphrodite	CON	27	609
		PD	25	576
	Male	CON	3	90
		PD	5	144
mut-16(pk710)	Hermaphrodite	CON	5	151
		PD	4	116
	Male	CON	5	103
		PD	4	76

Table 2. Number of biological replicates and sample sizes for pheromone avoidance assays. Biological replicates represent assays set up independently. Sample size represents the total number of animals tested for a specific genotype.

the total number of animals tested can be found in Table 2. The number of sample sizes of CON and PD within each category were approximately matched to meet the assumption of equal variance between samples, reducing type I error. To compare avoidance indices of CON and PD worms, multiple *t*-tests followed by Holm-Sidak correction were performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

2.2.5 *osm-9* expression levels

This experiment was conducted by Nikolaus Wagner. The expression levels of *osm-9* were measured using an integrated transgene array expressing GFP under the regulation of 350 bp of *osm-9* upstream regulatory sequences as previously described (*pdrIs1*) (Sims *et al.* 2016). This integrated array was genetically crossed into *mut-16(mg461)* background. The expression of GFP was determined in control and postdauer adults 24 hours after the L4 larval stage. The worms were dye-filled using DiD, a stain that labels a group of amphid sensory neurons including ADL, to identify the location of ADL. The worms were mounted on agar pads with sodium azide and imaged using a Leica DM5500B microscope with a Hamamatsu 741 camera controller C10600 ORCA-R2 as described (Sims *et al.* 2016). At least 3 biologically independent trials were performed per condition and genotype, with at least 44 animals tested for each genotype and condition. To compare the proportion of animals expressing GFP in ADL neurons of hermaphrodite and male worms, multiple *t*-tests followed by Holm-Sidak correction were performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

2.3 Results

2.3.1 Outcrossing rates in N2 Bristol populations are dependent on the developmental history of the males

To investigate the molecular mechanisms that promote outcrossing in postdauer populations, we first investigated whether the common *C. elegans* lab strain, N2 Bristol, exhibits an increased outcrossing frequency after passage through dauer as observed previously for other wild isolates (Morran *et al.* 2009a). We performed mating assays with all combinations of CON and PD, male and hermaphrodite adults. The outcrossing frequency was calculated using the proportion of male progeny as an estimate of the outcrossing rate as previously described (Morran *et al.* 2009a; see Chapter 2.2.2). For populations of control hermaphrodites and control males, we observed an average male frequency of 0.172 within the progeny, serving as the baseline of outcrossing (Fig. 6). When either sex experienced dauer (males or hermaphrodites), we observed a slight, albeit nonsignificant, increase in outcrossing rates (0.293 and 0.209, respectively) (p = 0.129, 0.887, respectively, two-way ANOVA with Tukey's *post-hoc* test) (Fig. 6). However, when both sexes had experienced dauer, we observed a significant increase in outcrossing (0.350) compared to the population of control adults (p = 0.012, two-way ANOVA with Tukey's *post-hoc* test) (Fig. 6). We found that the type of males (CON or PD), but not hermaphrodites, had a significant effect on outcrossing levels in N2 Bristol (p = 0.0016, two-way ANOVA) (Table 6), indicating that the developmental history of males is the major determinant of outcrossing rates in wild-type populations (Fig. 6). However, when comparing the outcrossing assays where only males experienced dauer to the assays where neither parent experienced dauer, no significant differences were

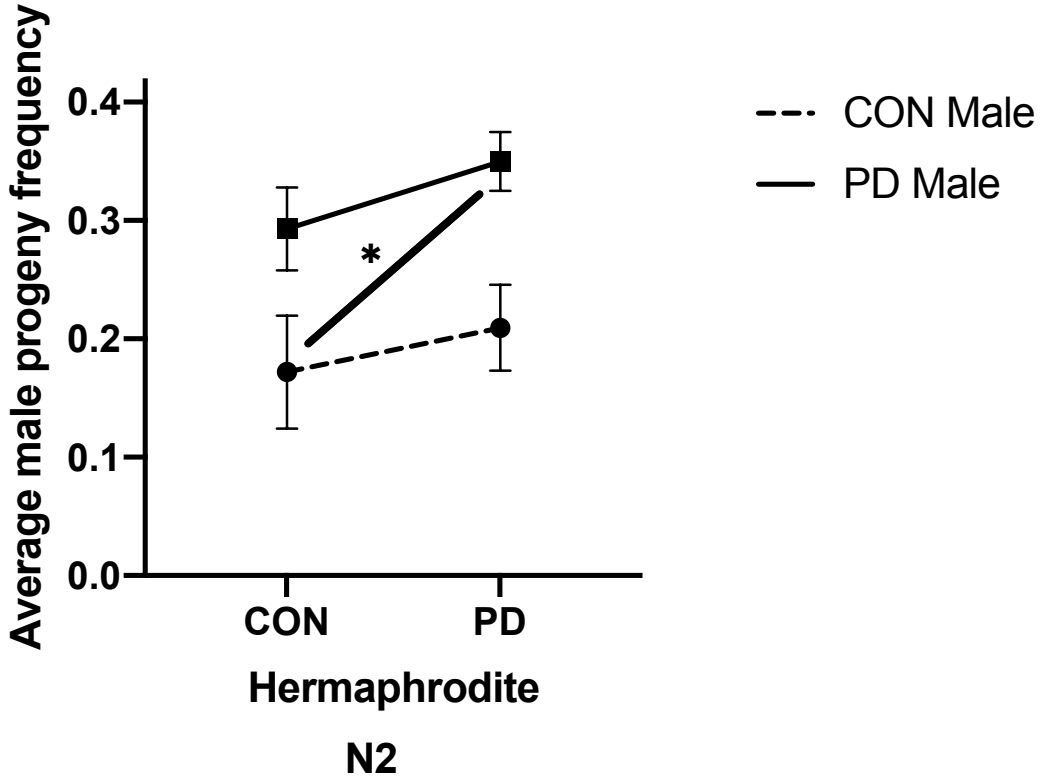


Figure 6: N2 exhibits increased outcrossing following dauer diapause. Mean outcrossing rates ± SEM for N2 Bristol. The dashed lines denote CON males, and the solid lines denote postdauer males. The x-axis denotes CON and PD hermaphrodites. The number of independent trials (N) and number of animals (n) are N ≥ 8 trials, n = 408 animals. Two-way ANOVA with Tukey's *post-hoc* test, * $p < 0.05$.

observed (Fig. 6). This suggests a minor role for the hermaphrodite's developmental history in promoting facultative outcrossing following dauer diapause. Our results have demonstrated that postdauer populations of N2 Bristol also exhibit increased outcrossing similar to the CB4856 isolates reported previously (Morran *et al.* 2009a).

2.3.2 Expression of *osm-9* is regulated in a sex-specific manner

Since our original hypothesis was that altered pheromone responses resulting from changes in *osm-9* expression underlie increased postdauer outcrossing rates, we

next examined the regulation of *osm-9* in males. Using a strain carrying an integrated GFP reporter transgene driven by ~350 bp of the *osm-9* promoter (*osm-9*p*::gfp*), we quantified the number of ADL neurons that express GFP in control and postdauer wild-type hermaphrodites and males. As expected, we observed that significantly fewer postdauer hermaphrodites have *osm-9*p::*gfp* expression compared to controls (Fig. 7A). However, in males, *osm-9* is expressed in ADL neurons in both control and postdauer adults (Fig. 7A). We showed previously that hermaphrodites carrying a mutation in *mut-16* exhibited increased expression of GFP in postdauer hermaphrodite ADL neurons compared to wild-type (Sims et al. 2016). MUT-16 is a glutamine/asparagine motif-rich protein that is required for the formation of the *Mutator* focus, a phase-separated perinuclear condensate important for endogenous siRNA amplification (Zhang *et al.* 2011; Phillips *et al.* 2012; Uebel *et al.* 2018). To examine if MUT-16 also plays a role in the regulation of *osm-9* expression in males, we examined the GFP expression in ADL neurons in control and postdauer males in the *mut-16* strains. We observed that *osm-9* continued to be expressed in control and postdauer males in the *mut-16* strain similar to wild-type (Fig. 7A).

Avoidance of ascr#3 is an ADL-mediated, *osm-9* dependent behavior in hermaphrodites (Jang et al 2012). We demonstrated previously that postdauer hermaphrodites fail to avoid high concentrations of ascr#3 due to the downregulation of *osm-9* in their ADL neurons (Sims *et al.* 2016). To examine the ascr#3 avoidance behavior in postdauer animals, we tested the response of wild-type and *mut-16* control and postdauer hermaphrodites and males to 100 mM ascr#3 using a drop test assay, as well as M13 buffer (negative control) and 1M glycerol (positive control) (Fig. 7B;

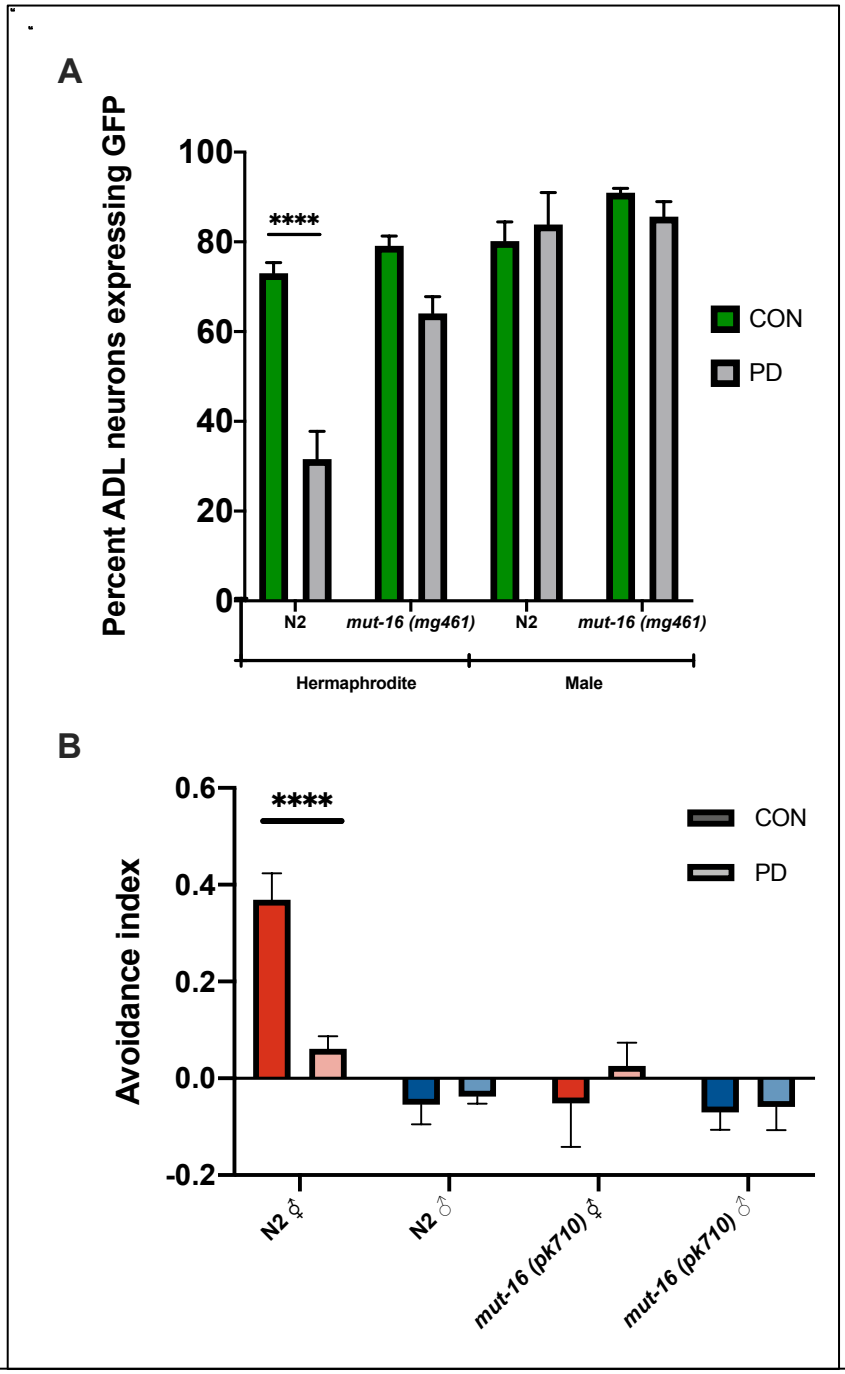


Figure 7: *osm-9* is regulated in a sex-specific manner. (A) Mean percent of ADL neurons expressing GFP ± SEM in an N2 Bristol and *mut-16*(*mg461*) background. The number of independent trials (N) and number of animals (n) are: N ≥ 3 trials, n ≥ 44. (B) The avoidance index ± SEM for N2 Bristol hermaphrodites and males in response to 100mM ascr#3 normalized to M13 avoidance. The number of independent trials (N) and number of animals (n) are: N ≥ 3 trials, n ≥ 93 animals. Multiple *t*-tests, **** $p < 0.0001$. Data in A were collected by Nikolaus Wagner.

Appendix 5.4). Since *osm-9* expression was not significantly different between control and postdauer males in ADL neurons, a difference in their ascr#3 avoidance indices would suggest that ascr#3 avoidance is independent of *osm-9* expression in ADL neurons in males. While the control chemicals elicited the expected behaviors in both control and postdauer, N2 and *mut-16(pk710),* males, ascr#3 did not elicit a difference in avoidance behavior in postdauer males compared to control males (Fig. 7B). This result indicates that the differential regulation of *osm-9* due to passage through the dauer stage is sex-specific.

2.3.3 TGF-β signaling pathway is required for increased outcrossing in postdauer populations

We previously showed that the *osm-9* TRPV channel gene is regulated by the TGF-β pathway in ADL neurons of postdauer hermaphrodites. Specifically, the DAF-3/co-SMAD transcription factor binds to the *osm-9* promoter to promote its downregulation after dauer, and mutations in *daf-3* result in continued *osm-9* expression and ascr#3 avoidance in postdauer hermaphrodites (Sims *et al.* 2016). Thus, we tested whether the increased outcrossing levels in postdauer populations were dependent upon TGF-β signaling by performing mating assays using a *daf-3(mgDf90)* mutant strain. For mating assays of *daf-3* control hermaphrodites and control males, we observed a similar baseline of outcrossing to what we observed for wild-type (Fig. 8A). Although we did observe a slight increase in outcrossing when the *daf-3* mating assays included postdauer males, postdauer hermaphrodites, or both, the progeny male frequencies were not significantly different from baseline (p = 0.168, 0.480, and 0.128,

respectively; Fig. 8A). These results suggest that DAF-3 and the TGF-β signaling pathway are required for increased outcrossing in postdauer populations.

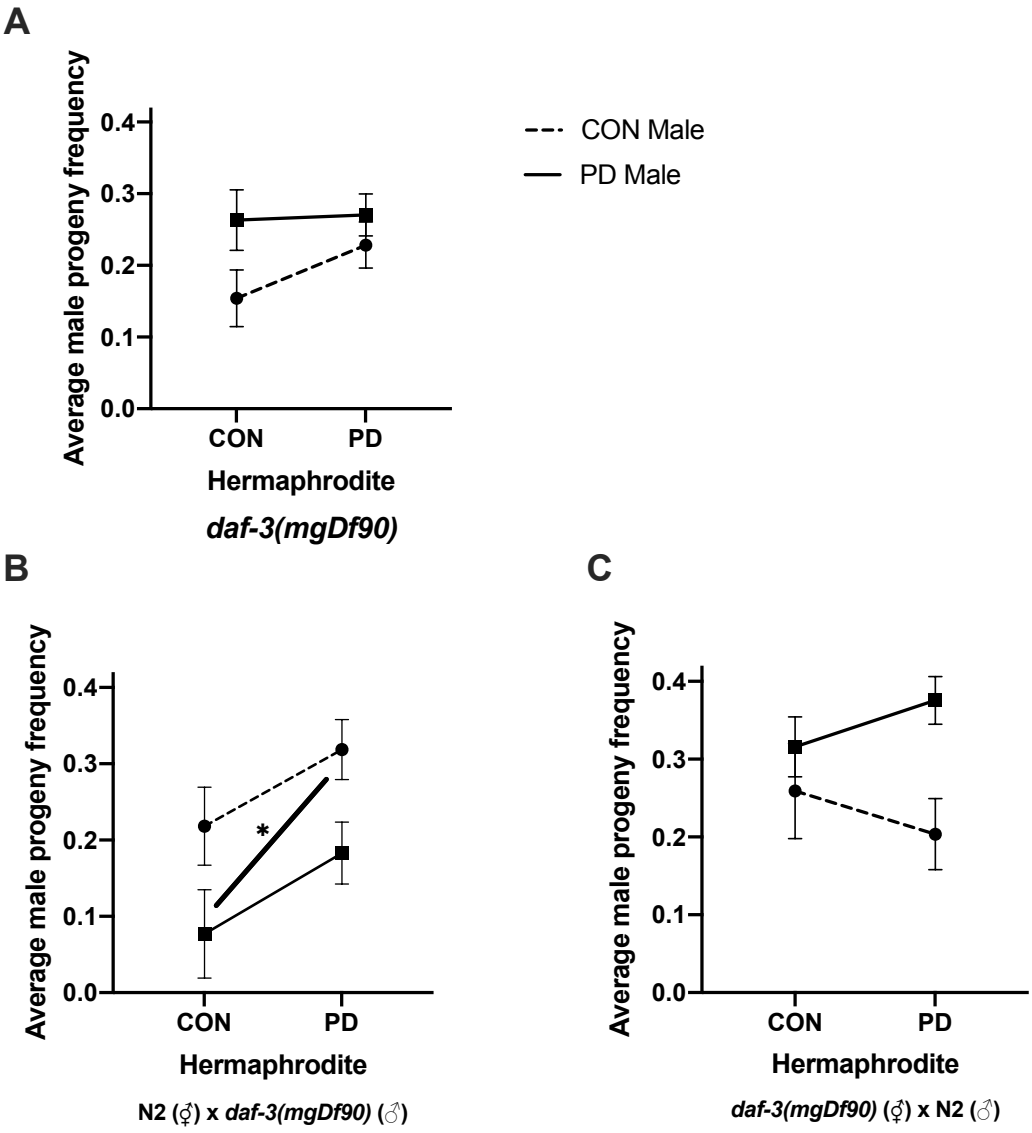


Figure 8: TGF-β signaling pathway plays a role in increased outcrossing rate. Mean outcrossing rates ± SEM for (A) *daf-3*(*mgDf90*), (B) N2 Bristol hermaphrodites mated with *daf-3* males, and (C) *daf-3* hermaphrodites mated with N2 Bristol males. The dashed lines denote CON males, and the solid lines denote postdauer males. The x-axis denotes CON and PD hermaphrodites. The number of independent trials (N) and number of animals (n) for a given cross are A*:* N = 8 trials, n = 364 animals, B: N ≥ 3 trials, n = 180 animals, and C: N = 4 trials, n = 192 animals. Two-way ANOVA with Tukey's *post-hoc* test, * $p < 0.05$.

We next asked whether DAF-3 was required in males or hermaphrodites to promote postdauer outcrossing. We predicted that the continued expression of *osm-9* and ascr#3 avoidance in postdauer *daf-3* hermaphrodites would decrease outcrossing. Alternatively, DAF-3 may be required in postdauer males to promote outcrossing, as suggested by our wild-type outcrossing results (Fig. 6). To distinguish between these possibilities, we examined male frequencies in the progeny of populations where only one sex carrying a mutant allele of *daf-3* was mated with wild-type animals of the other sex. In populations where *daf-3* males were mated to wild-type hermaphrodites, we found that the trends in outcrossing were the opposite compared to wild-type. Interestingly, mating assays that included postdauer wild-type hermaphrodites and control *daf-3* males exhibited significantly higher male progeny frequencies compared to mating assays with control wild-type hermaphrodites and postdauer *daf-3* males (p = 0.016; Fig. 8B). These results are consistent with both a requirement for DAF-3 in postdauer males to promote outcrossing and that the type of hermaphrodites (CON or PD) plays a role in outcrossing levels. We further investigated a potential role of DAF-3 in hermaphrodites by examining the male progeny frequency in mating assays of *daf-3* hermaphrodites with wild-type males. If hermaphrodites did not play a significant role in outcrossing levels, we would expect that this assay would look similar to the wild-type mating assays. Surprisingly, all combinations of control and postdauer populations did not show significant differences in male progeny frequencies, supporting the hypothesis that DAF-3 also plays a role in hermaphrodites to promote postdauer outcrossing (Table 6; Fig. 8C). This change in the outcrossing frequency trend can be attributed to an increase, albeit nonsignificant, in the baseline (CON-H x CON-M) for male frequency in figure 8C compared to that of N2 Bristol. Together, these results indicate that DAF-3 is

required in both males and hermaphrodites to promote outcrossing in postdauer populations.

2.3.4 RNAi pathway regulates mating frequency

In addition to the requirement of the TGF-β signaling pathway, the endogenous RNAi pathway is also required for the downregulation of *osm-9* in postdauer hermaphrodite ADL neurons (Sims *et al.* 2016). Thus, we tested the hypothesis that RNAi is also required for increased outcrossing in postdauer populations by performing mating assays using the *mut-16(pk710)* mutant strain. In the mating assay of *mut-16* control hermaphrodites and control males, we found a male progeny frequency similar to what was observed for wild-type. However, we found that the male progeny frequencies from all mating assay combinations of *mut-16* control and postdauer adults were not significantly different and were similar to the baseline outcrossing levels of wild-type control populations (Fig. 9A). These results indicate that MUT-16 is required for the increased outcrossing phenotype in postdauer populations.

In previous work, we demonstrated that *mut-16* animals were dauer deficient in the presence of high pheromone levels due to decreased expression of genes encoding G proteins required for neuronal signaling (Bharadwaj and Hall 2017). In addition, we were able to rescue, at variable levels, the dauer deficient larval phenotype by expressing a high-copy transgene array carrying wild-type *mut-16* coding sequence either pan-neuronally or in individual, pheromone-sensing neurons (Bharadwaj and Hall 2017). To determine if MUT-16 was also functioning in neurons to regulate mating behaviors, we performed the mating assay using a *mut-16* strain carrying a rescue transgene driven by a pan-neuronal promoter (*rab-3*p). Interestingly, we found that male progeny

frequencies were suppressed below baseline outcrossing in the *mut-16* pan-neuronal rescue strain compared to *mut-16* mutants (Fig. 9A and 9B).

Next, we attempted to perform mating assays using *mut-16* strains that carry a rescue transgene driven by single-neuron promoters. We examined the male progeny frequencies of *mut-16* strains rescued only in ADL neurons (*sre-1*p) and found that this

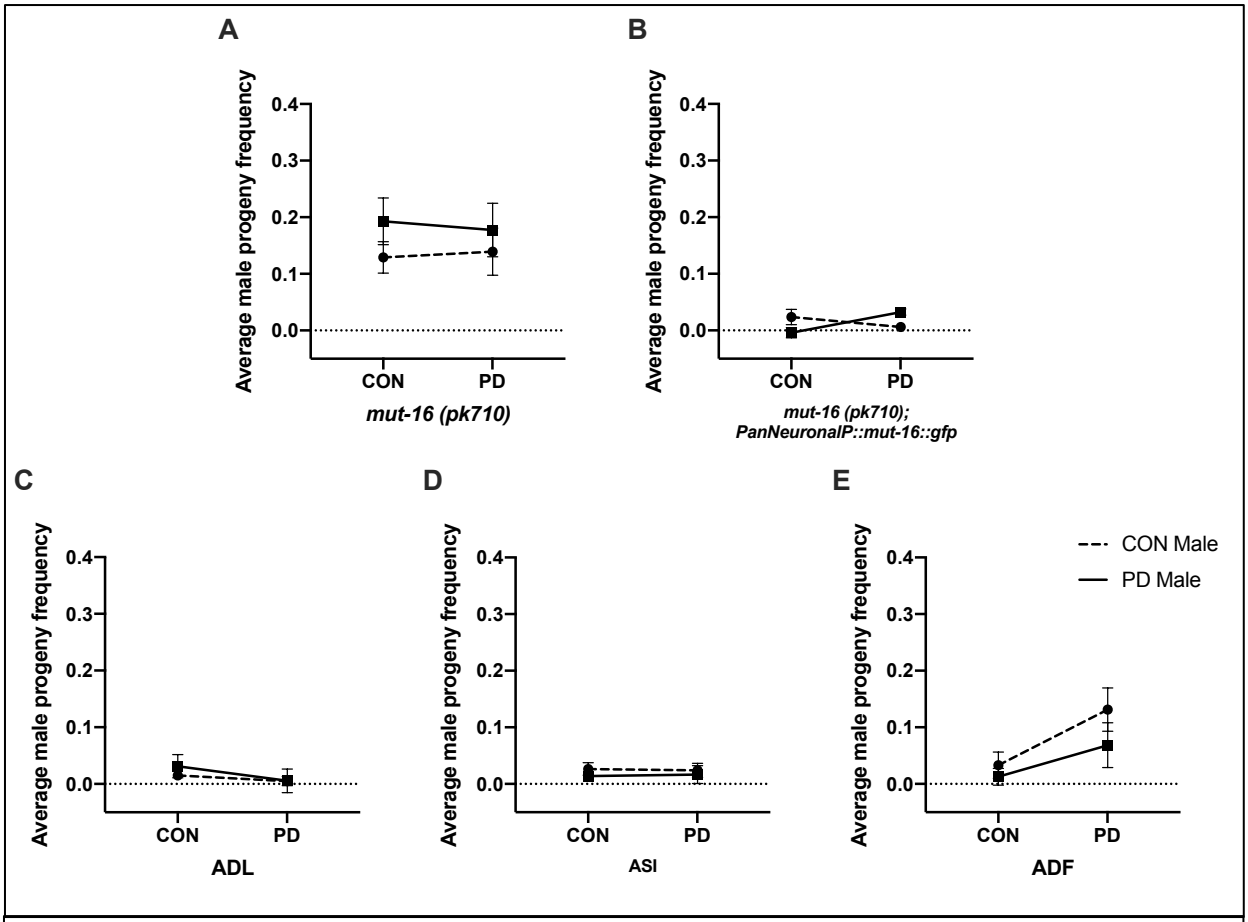


Figure 9: RNAi pathway is required for increased outcrossing. Mean outcrossing rates ± SEM for (A) *mut-16(pk710),* (B) *mut-16(pk710); rab-3*P*::mut-16::gfp* (pan-neuronal rescue), (C) ADL, (D) ASI, and (E) ADF neuronal specific rescues. Both B and D are integrated rescue lines. The dashed lines denote CON males, and the solid lines denotes postdauer males. The x-axis denotes CON and PD hermaphrodites. The number of independent trials (N) and number of animals (n) for a given cross are *mut-16(pk710)*: N = 3 trials, n = 134 animals, *mut-16(pk710); PanNeuronal*P*::mut-16::gfp :* N = 3 trials, n = 140 animals, ASI: N = 3 trials, n = 134 animals, ADL: N ≥ 3 trials, n = 116 animals and ADF: N = 5 trials, n = 232 animals. Two-way ANOVA with Tukey's *post-hoc* test.

strain also exhibited suppressed outcrossing below baseline (Fig. 9C). We observed similar suppressed outcrossing levels for the *mut-16* strains rescued in the pheromone-sensing neurons ASI (*gpa-4*p) and ADF (*srh-142*p) (Fig. 9D and 9E). These results support the conclusion that *mut-16* expression is required, at endogenous levels, for promoting outcrossing in postdauer populations. One explanation for the suppressed outcrossing levels in the strains overexpressing *mut-16* was the observation that the total brood size of the mating assays was significantly less than *mut-16* or wild-type strains. This phenotype is explored further in Chapter 3.

2.3.5 Postdauer males exhibit increased ability to detect mates via pheromone

Recent work has shown that males find mates through detection and chemotaxis towards pheromone molecules produced by hermaphrodites (Srinivasan *et al.* 2008; Leighton *et al.* 2014; Fagan *et al.* 2018). Our results indicate that the developmental history of the male is a significant contributor to outcrossing rates, with hermaphrodites playing a more minor role. We hypothesized two possible, non-mutually exclusive, mechanisms to explain how postdauer males contribute to outcrossing levels. First, postdauer males may be more efficient at finding mates than control males. A second possibility is that postdauer males may produce a novel pheromone component that functions as an attractant to hermaphrodites.

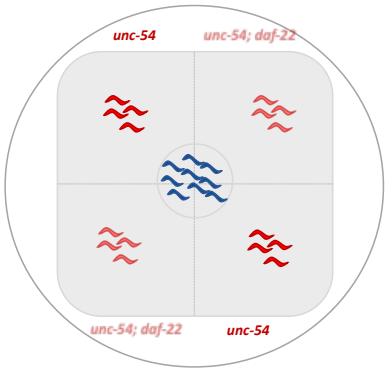
To test these possibilities, we first performed mate preference assays using genetically immobilized hermaphrodites to determine male preference and ability to locate mates (Fagan *et al.* 2018; see Chapter 2.2.3). Each quadrant contained immobilized control hermaphrodites that can (*unc-54*) or cannot (*unc-54; daf-22*)

secrete pheromones into their environment. In each assay, either control or postdauer wild-type males were placed in the center of the plate, and after 30, 60, and 90 minutes were scored for their location in the quadrants (Fig. 10A). First, both control and postdauer males exhibited preference towards hermaphrodites that were able to secrete pheromones (Fig. 10B). Interestingly, we observed that postdauer males were better able to locate the pheromone-producing hermaphrodites compared to control males (Fig. 10B). These results support the hypothesis that postdauer males are more efficient at locating mates compared to control males.

To test the possibility that postdauer males secrete a pheromone for hermaphrodite attraction, we performed the same chemotaxis quadrant assays using immobilized (*unc-54*) control or postdauer males in each quadrant. Control wild-type hermaphrodites were allowed to roam on the plate and were scored every 30 minutes, up to 90 minutes (Fig. 10C). In contrast to the assay for male preference, the hermaphrodites did not exhibit any preference between control and postdauer males (Fig. 10D). We also conducted chemotaxis quadrant assays with either 10mM ascr#3 or 100mM ascr#3 to test if the increased mate locating ability was facilitated by detection of ascr#3 (Fagan *et al.* 2018; see Chapter 2.2.3). Males were tested for their preference to four quadrants, two opposing quadrants containing either ascr#3 or a negative control (Fig. 10E). Control and postdauer males did not exhibit a difference in chemotaxis towards ascr#3 at the 10mM concentration (Fig. 10F). However, while statistically insignificant, there was a slight increase in the chemotaxis index for postdauer males at 100mM ascr#3 concentration (Fig. 10F). This is to be expected because while ascr#3 is playing a role in attracting postdauer males more efficiently, it is likely not the only pheromone component responsible for this, but there is likely a

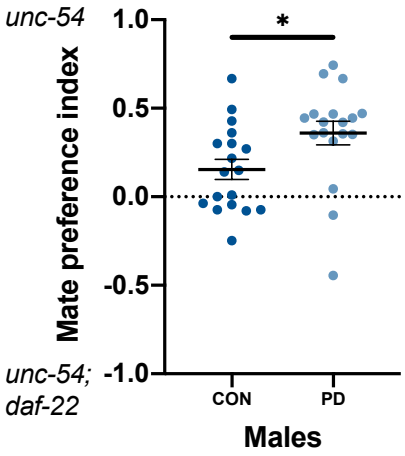
different blend of pheromones that is unique to postdauer hermaphrodites, making them more attractive to males. Together, these results suggest that postdauer males promote outcrossing through their increased ability to locate potential mates using pheromone cues.

A

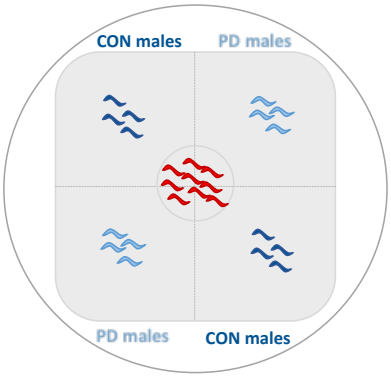


Mate preference index: $\frac{unc-54 - unc-54;daf-22}{unc-54 + unc-54;daf-22}$

B

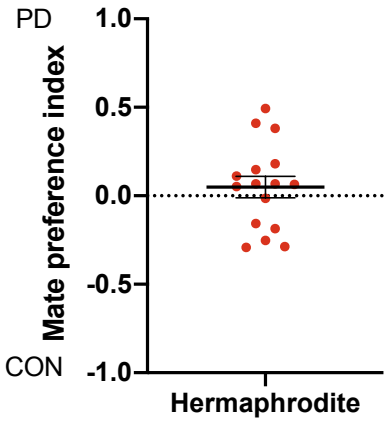


C

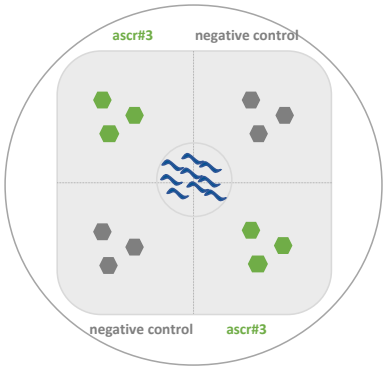


Mate preference index: $\frac{\text{PD male} - \text{CON male}}{\text{PD male} + \text{CON male}}$

D



E



Chemotaxis index: $\frac{\text{ascr\#3} - \text{negative control}}{\text{ascr\#3} + \text{negative control}}$

F

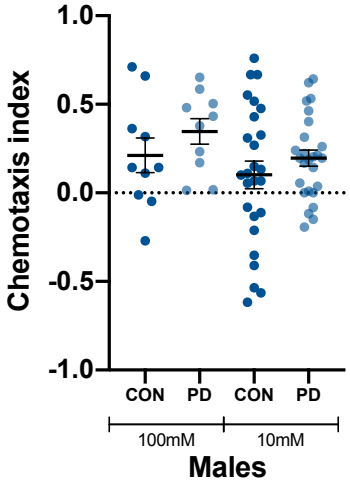


Figure 10: Males exhibit an increased ability to locate mates following early environmental stress. A, C, and E are schematic of the mate preference (A and C) and chemotaxis (E) assay experimental setup. Immobilized hermaphrodites of the same genotype were placed on opposite quadrants and males were scored based on quadrants they are found in. The chemotaxis index ± SEM for *him-5* males (CON or PD, blue and light blue, respectively) in response to immobilized hermaphrodites (B) or ascr#3 (F) and hermaphrodites (red) in response to immobilized males (D). The number of independent trials (N) and number of animals (n) for B: N = 18 trials, n = 180 animals, D: N = 16 trials, n = 160 animals, E: N ≥ 10 trials, n ≥ 100 animals. Unpaired *t*-test, * $p < 0.05$. Data in this figure are collected by Dr. Jintao Luo.

2.4 Conclusions

Here, we show that the change in reproductive strategies in *C. elegans* following exposure to an adverse environment requires sexually dimorphic changes in behavior. We found that postdauer males are better able to locate hermaphrodites compared to continuously developed males, thus promoting increased outcrossing. The change in the outcrossing rate following the dauer diapause is dependent on both RNAi and TGF-β signaling pathways. Mutants in both pathways lose the increased outcrossing phenotype in postdauer animals. Thus, sexually dimorphic changes in behavior mediate increased outcrossing, allowing for postdauer populations to harvest the benefits of that particular mating mode.

We observed sex-specific regulation of *osm-9*; unlike in postdauer hermaphrodites, *osm-9* is not downregulated in postdauer males, and no significant change in ascr#3 attraction or avoidance was observed in postdauer males compared to controls. Overall, we find that the ADL-specific, differential expression of *osm-9* in hermaphrodite postdauers contributes to the increased facultative outcrossing following exposure to starvation stress during early development. Neuronal rescues of *mut-16*

activity do not restore mating behavior. In fact, the overexpression of *mut-16* either pan-neuronally or in specific chemosensory neurons leads to a complete abolishment of mating.

Chapter 3: The effects of RNAi pathway on fecundity and gonad morphology

3.1 Introduction

MUT-16, a glutamine/asparagine motif-rich protein, is required for the formation of the *Mutator* focus, a phase-separated perinuclear condensate in the germline located adjacent to similar structures including the P granule, the Z granule, and the SIMR focus (Brangwynne *et al.* 2009; Phillips *et al.* 2012; Uebel *et al.* 2018; Manage *et al.* 2020). MUT-16 is essential for the biogenesis and amplification of siRNAs in the RNAi pathway (Zhang *et al.* 2011; Phillips *et al.* 2012; Uebel *et al.* 2018).

RNAi has been shown to be important for dauer formation, which can be induced by high pheromone concentrations. Previous work from the Hall laboratory showed that *mut-16* hermaphrodites exhibited a decrease in the expression of G proteins, which are required in their chemosensory neurons to respond to environmental cues (Bharadwaj and Hall 2017). Thus, the *mut-16* animals lost their ability to detect dauer formation pheromones and respond appropriately. A similar mechanism is likely responsible for the requirement of RNAi for mating behavior, given that mating occurs through a pheromone-based mechanism. However, while a pan-neuronal rescue transgene of *mut-16* was capable of restoring the dauer formation phenotype in larvae, we showed in Chapter 2 that the same strain does not restore mating behavior deficits of *mut-16* animals. In fact, the overexpression of *mut-16* either pan-neuronally or in specific chemosensory neurons led to a complete abolishment of mating. In this chapter, we investigated the mechanisms responsible for this mating decline in *mut-16* rescue strains.

The suppressed outcrossing levels in the *mut-16* neuronal rescues could be attributed to either a significant decrease in mating events, the inability to mate, or defects in the germline. Since the mutant strain *mut-16(pk710)* is known to exhibit a decreased brood size compared to wild-type animals (Zhang *et al.* 2011; Rogers and Phillips 2020), we examined the ability of the *mut-16* pan-neuronal rescue strains to produce self-fertilized progeny and found that they exhibited significantly reduced fecundity, to near sterility, compared to wild-type and *mut-16* strains. This sterility is due to small germlines and low germ cell counts in the rescue strains that are transgenerationally inherited in the absence of the rescue transgene. The results in this chapter indicate that overexpression of *mut-16* in neurons results in severe fecundity and gonad defects in adults.

3.2 Materials and methods

3.2.1 Brood size assays

The strains used for brood size assays in this section are listed in Appendix 5.1. For the regular brood size assays, L4 hermaphrodites were placed on seeded NGM plate and transferred to new plates daily until egg-laying ceased, and the number of surviving progeny per plate was determined. For the transgenerational brood size assay, ten hermaphrodites carrying the extrachromosomal array overexpressing *mut-16* pan-neuronally were used to establish the parent plate (P_0) (Fig. 11A). Such extrachromosomal arrays are known to express genes at higher levels compared to endogenously expressed genes due to the multicopy nature of the arrays (Nance and Frøkjær-Jensen 2019). A red fluorescent co-injection marker was used when generating these *mut-16* rescue strains, thus RFP+ refers to progeny that inherited the

extrachromosomal rescue array, while RFP- refers to the animals that did not inherit the array (Bharadwaj and Hall 2017). Ten hermaphrodite progeny (either RFP+ or RFP-) were then transferred to a new NGM plate to establish F1 and subsequent generations, and another set of ten was used to set up the brood size assays, with one hermaphrodite per plate. The progeny on the brood size assay plates were counted once they reach the L4/adult stage. To compare the brood sizes over the different generations of *mut-16* pan-neuronal rescues, multiple *t*-tests followed by Holm-Sidak correction were performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

3.2.2 DAPI staining

A standard DAPI staining protocol for whole worms was used (Francis *et al.* 1995; Qiao *et al.* 1995). Worms were picked at the L4 stage then DAPI stained approximately one day later, at the adult stage. The worms were first fixed in 100% cold methanol, then washed with 0.1% PBST. DAPI staining was done using a 1:1000 dilution of the stain, then the worms were mounted on glass slides and imaged using a Leica DM5500B microscope with a Hamamatsu 741 camera controller C10600 ORCA-R2.

3.2.3 Germline morphology scoring

Germline scoring was performed by Alexandra Nichitean. The whole-mount DAPI stained images of each gonad were visually scored based on several criteria, with animals given a score of zero or one based on the absence or presence of each criterion. The different categories were as follows: DTC migration defects, egg-laying defects, gamete number, abnormal gonad morphology, and germline defects. Animals scored for

the presence of DTC migration defects include weak to severe meandering on dorsal pathfinding defects (Wong and Schwarzbauer 2012). The young adult hermaphrodites were scored for potentially having an egg-laying defect if they had a greater number of eggs retained within the uterus in a disorganized manner at the time of staining compared to wild-type (Soto *et al.* 2002). Animals were scored as having a gamete number defect by visually comparing the amount of sperm present between wild-type and mutant animals, which included low to no sperm present. Within the abnormal gonad morphology category, animals were marked for having a smaller overall gonad size and a thinner appearance of the distal gonad compared to wild-type nematodes.

Genotype	Array state	Number of animals	Number of gonads
N2	n/a	22	43
mut-16(pk710)	n/a	31	55
mut-16(pk710); PdrEx64 F1	RFP+	14	25
	RFP-	18	35
mut-16(pk710); PdrEx64 F3	RFP+	16	31
	RFP-	27	52
mut-16(pk710);PdrEx64 F5	RFP+	24	46
	RFP-	25	48

Table 3. Number of animals and gonads quantified for germline morphology scoring. Each genotype and category had 3 biological replicates performed. Sample size represents the total number of animals quantified. Number of gonads represent the total number of gonad arms quantified for a specific genotype.

Germline defects were quantified by counting the total germ cell nuclei for each gonad arm using the multi-point tool in ImageJ (NIH). The germ cell nuclei were further classified into mitotic, transition, and pachytene zones by identifying the characteristic germ cell morphology. The transition and pachytene zone were deemed to start when at least two cells in a row showed the crescent-shape and basket-shape nuclei morphology, respectively (Shakes *et al.* 2009). A total of 3 biological replicates were performed. The number of gonads and total animals examined can be found in Table 3. To compare the different classes of germline morphology defects, one-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 8.4.3 for macOS, GraphPad Software, San Diego, California USA.

3.3 Results

3.3.1 Neuronal overexpression of *mut-16* negatively influences fecundity

While performing the outcrossing assays with the *mut-16* neuronal rescue strains, we observed that these strains exhibited a decrease in total progeny compared to other strains. To investigate if the suppressed outcrossing levels were due to germline defects, we first examined the ability of the *mut-16* neuronal rescue strains to produce self-fertilized progeny. We conducted brood size assays using N2 Bristol, *mut-16*(*pk710*), and two independent lines of the *mut-16* strain carrying an extrachromosomal array of the *mut-16* rescue transgene driven by a pan-neuronal promoter (*rab-3*p). First, we observed that wild-type postdauer hermaphrodites showed a significantly reduced brood size compared to controls, and that this difference is eliminated in the *mut-16(pk710)* strain as we reported previously (Fig. 11) (Hall *et al.*

2010; Ow *et al.* 2018). In addition, we found that control and postdauer adults of both pan-neuronal *mut-16* rescues exhibited a significant reduction in brood size compared

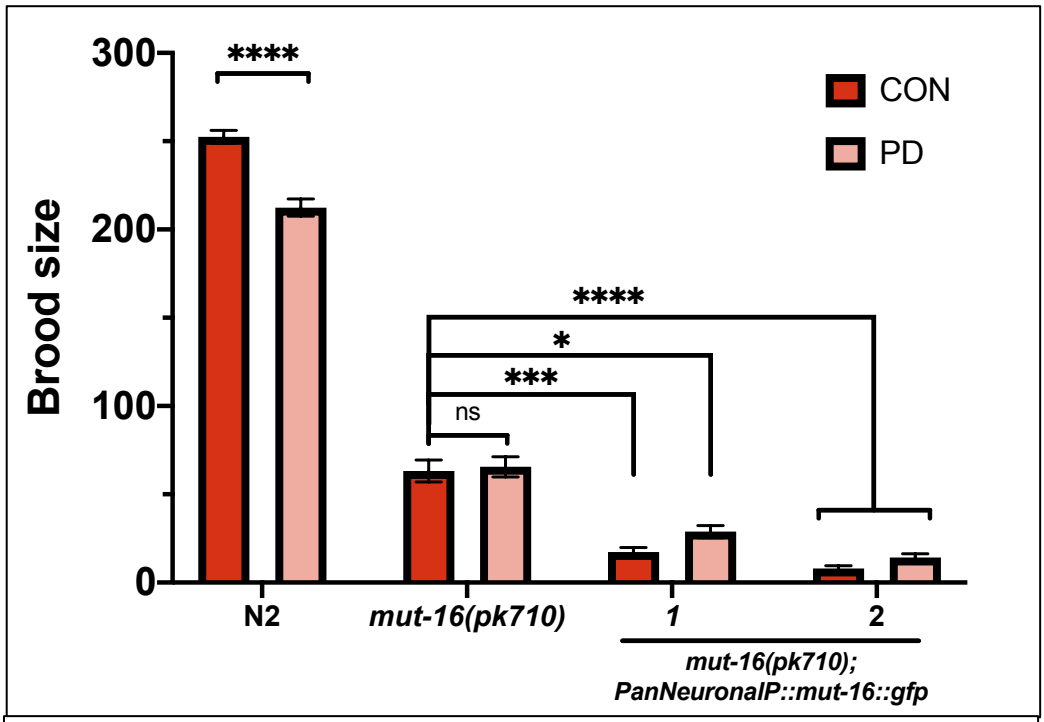


Figure 11: Neuronal overexpression of *mut-16* decreases fecundity. Mean brood size ± SEM in an N2 Bristol *mut-16*(*pk710*)*, mut-16*(*pk710*)*; rab-3*P*::mut-16::gfp* (pan-neuronal rescue). The number of independent trials (N) and number of animals (n) are: N ≥ 4 trials, n ≥ 31. One-way ANOVA with Tukey's *post-hoc* test, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Data in this figure are collected by Dr. Pallavi Bharadwaj and Alexandra Nichitean.

to wild-type and *mut-16* strains (Fig. 11), indicating that the low fecundity of the rescue strains was not merely due to the *pk710* allele alone. Thus, overexpression of *mut-16* in neurons has negative consequences on fecundity in *mut-16* mutants.

3.3.2 The negative effects on fecundity are transgenerationally inherited

Double-stranded RNA (dsRNA), the trigger for RNAi, has been shown to enter the germline and regulate gene expression transgenerationally (Fire *et al.* 1998; Grishok *et al.* 2000; Winston *et al.* 2002; Jose and Hunter 2007; Devanapally *et al.* 2015). Thus,

we questioned whether the reduced brood size observed in the MUT-16 pan-neuronal rescue strains could be inherited over generations in the absence of the *mut-16* rescue transgene. To address this question, we used a *mut-16* strain carrying an extrachromosomal array of the *mut-16* rescue transgene. We counted the brood sizes of F1 to F5 generation progeny of animals that have retained (RFP+) or lost (RFP-) the

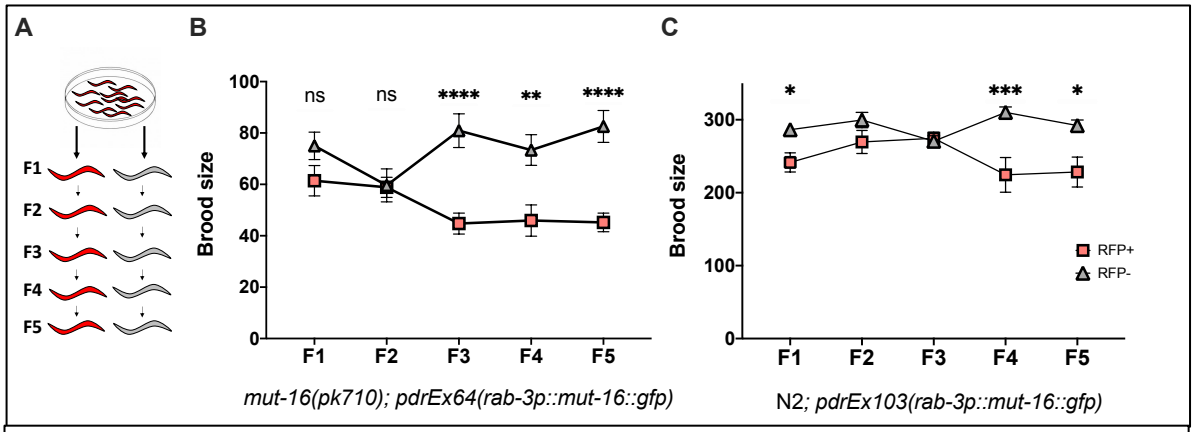


Figure 12: Pan-neuronal expression of *mut-16* affects fecundity transgenerationally. (A) Schematic of the brood size experimental setup. Mean brood size ± SEM for (B) *mut-16(pk710); rab-3*P*::mut-16::gfp* (pan-neuronal rescue), and (C) N2; *rab-3*P*::mut-16::gfp*. The x-axis denotes five consecutive generations of progeny from a parent carrying the extrachromosomal rescue array. The number of independent trials (N) and number of animals (n) for each generation are N ≥ 3 trials, n ≥ 28 animals. multiple *t*-tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (with exception for generations 4 and 5 for C, N = 2 trials, n ≥ 19 animals).

extrachromosomal *mut-16* transgene array in the F1 generation from an RFP+ hermaphrodite (Fig. 12A). The F1 and F2 generations did not exhibit a significant difference in brood size between RFP+ and RFP- populations. However, for the F3 through F5 generations, we observed that the RFP- populations had significantly larger brood size compared to the RFP+ counterparts (Fig. 12B). This result is due to a gradual decline in fecundity in the RFP+ progeny retaining the array, while RFP- progeny that lost the array maintained their brood size over the five generations.

We next asked if the decline in fecundity of the *mut-16* pan-neuronal rescue strains was due to accumulated mutations in a sensitized *mut-16*(*pk710*) background. Since *Mutator* foci can no longer form in the *mut-16*(*pk710*) background, the RNAi pathway is no longer able to suppress transposon activity, thus mutations accumulate rapidly in this strain (Ketting *et al.* 1999; Tabara *et al.* 1999; Sijen and Plasterk 2003). To address this question, we injected the wild-type, N2 Bristol strain, with the *rab-3*p*::mut-16::gfp* rescue transgene and examined the brood sizes of RFP+ and RFP- progeny of the F1 to F5 generations from an RFP+ hermaphrodite. For the F1 through F3 generations, the RFP+ populations show variable, but similar, brood sizes to the RFP- progeny. Starting in the F4 generation, we observed a similar decline of brood size in the RFP+ progeny retaining the array, while the RFP- progeny that lost the array maintained stable brood sizes (Fig. 12C). Together, these results suggest that the neuronal overexpression of *mut-16* is correlated with increased sterility over generations, possibly through inheritance of epigenetic factors such as small RNAs.

3.3.3 Neuronal overexpression of *mut-16* negatively influences gonad morphology transgenerationally

After observing the decrease in fecundity caused by overexpression of *mut-16* pan-neuronally, we investigated possible mechanisms leading to this phenotype in the *mut-16* neuronal rescues. Recently, *mut-16* mutants were shown to exhibit germline defects following heat stress such as small or collapsed gonad arms, defects in maintaining germ cell pluripotency, and misregulation of spermatogenic, oogenic, and somatic genes in the germline (Rogers and Phillips 2020). Thus, we asked whether neuronal overexpression of *mut-16* has a similar adverse impact on gonad morphology,

leading to decreased fecundity. To test this hypothesis, we examined the somatic gonad and germline morphology for defects in the F1, F3, and F5 generations of *mut-16* pan-neuronal rescue strain hermaphrodites that either retained (RFP+) or lost (RFP-) the overexpression array, comparing them to both N2 Bristol and *mut-16(pk710)* (see Chapter 3.2.3). Indeed, the proportion of *mut-16(pk710)* animals displaying overall gonad defects was significantly higher than that of N2 Bristol (Fig. 13A). The significant difference in gonad morphology between *mut-16(pk710)* and N2 can be attributed to DTC migration defects, low or no sperm, and thin distal gonads (Fig. 13B, E, and F). Since the decreased fecundity is more severe in animals retaining the array compared to *mut-16(pk710)* animals, we expected to observe a similar trend in the gonad morphology defects which would be exacerbated over generations when animals retain the *mut-16* neuronal overexpression arrays. In addition, since the significant decrease in brood size of RFP+ compared to RFP- progeny is established at F3, we expect the gonad phenotype that would explain the decreased fecundity to have a similar proportion of impacted animals at F1, but an increase in proportion exclusively for RFP+ progeny starting F3.

To test this prediction, we first examined DTC migration defects. We found that *mut-16(pk710)* had a significantly higher proportion of animals displaying defects compared to N2 Bristol (Fig. 13B). Additionally, the F3 and F5 generations of RFP+ progeny, and all RFP- generations had a significantly higher proportion of animals displaying DTC migration defects compared to N2 Bristol. However, none of the RFP+ or RFP- generations displayed a significant difference in the proportion of animals exhibiting the defects compared to *mut-16(pk710)* (Fig. 13B). While m*ut-16* does exhibit DTC migration defects, this phenotype is not worsened by the presence of the *mut-16* transgene (Fig. 13B). Similar to the DTC migration defects, the RFP+ and RFP- progeny

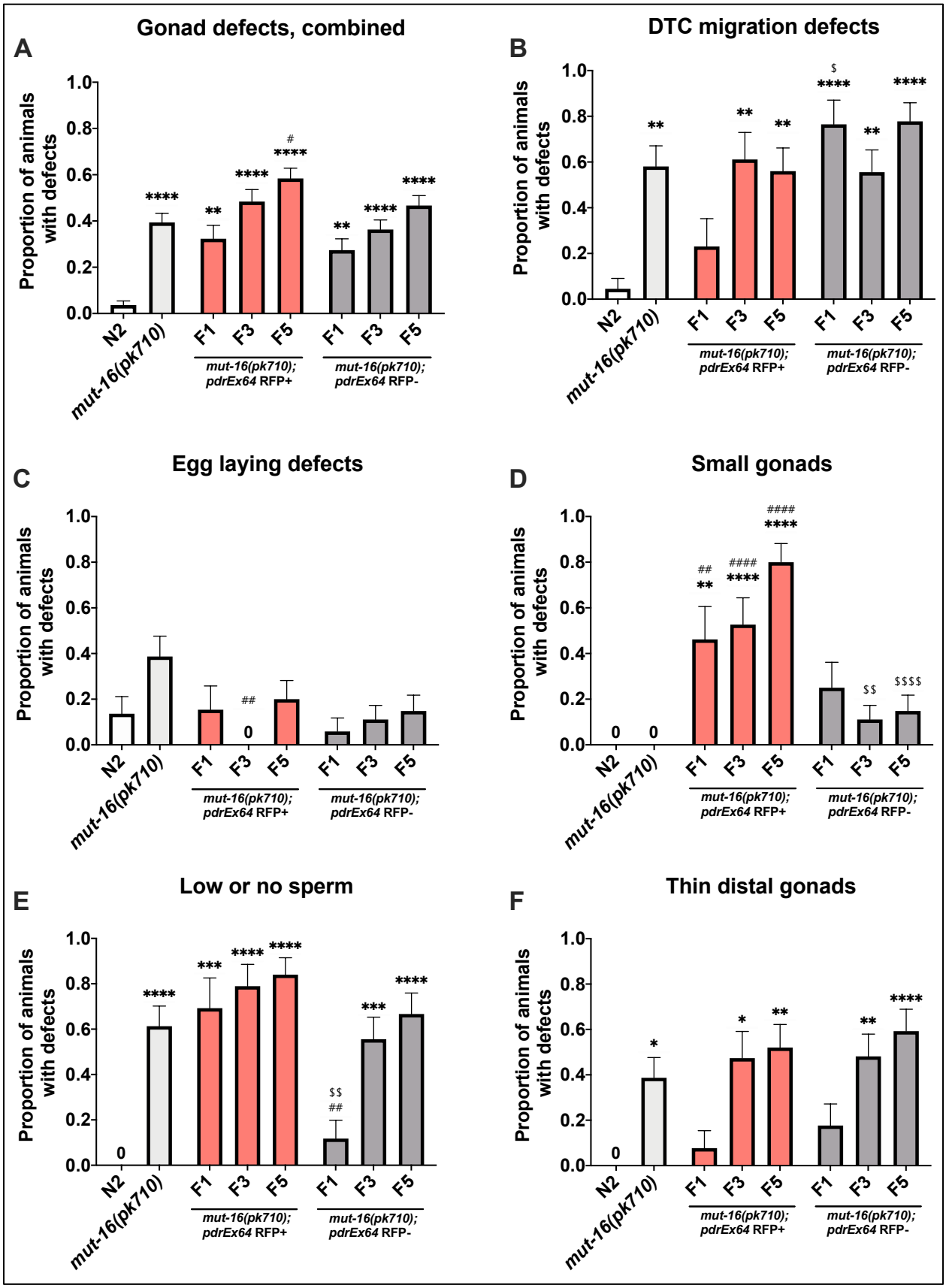

A
Gonad defects, combined
Proportion of animals with defects
1.0
0.8
0.6
0.4
0.2
0.0
****
**
****
#
****
**
****
****
N2
mut-16(pk710)
F1
F3
F5
F1
F3
F5
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-
B
DTC migration defects
Proportion of animals with defects
**
**
**
$
****
**
****
N2
mut-16(pk710)
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-
C
Egg laying defects
Proportion of animals with defects
##
0
N2
mut-16(pk710)
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-
D
Small gonads
Proportion of animals with defects
0
0
##
**
####
****
####
****
$$
$$$$
N2
mut-16(pk710)
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-
E
Low or no sperm
Proportion of animals with defects
0
****
***
****
****
$$
##
***
****
N2
mut-16(pk710)
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-
F
Thin distal gonads
Proportion of animals with defects
0
*
*
**
**
****
N2
mut-16(pk710)
mut-16(pk710); pdrEx64 RFP+
mut-16(pk710); pdrEx64 RFP-

Figure 13: Retention of *mut-16* neuronal overexpression array impacts somatic gonad morphology. Mean proportion of animals with germline defects ± SEM in N2, *mut-16(pk710), mut-16(pk710);pdrEx64[rab-3*P*::mut-16::gfp],* both RFP+ and RFP-. The germline defects categories shown are (A) combined gonad morphology; (B) DTC migration defects; (C) egg laying defects; (D) small gonads; (E) low or no sperm; and (F) thin distal gonads. The x-axis denotes the genotypes and the different consecutive generations of progeny from a parent carrying the extrachromosomal rescue array. The y-axis denotes the proportion of animals displaying the specific defect. The number of independent trials (N) and number of animals (n) for each genotype and generation are N = 3 trials, n ≥ 14 animals with at least 25 gonad arms examined. One-way ANOVA followed by Tukey's *post-hoc* test, *, #, $ $p < 0.05$, **, ##, $$ $p < 0.01$, ***, ###, $$$ $p < 0.001$, ****, ####, $$$$ $p < 0.0001$. * denotes a comparison to N2; # denotes a comparison to *mut-16(pk710),* $ denotes a comparison to RFP+ within the same generation. The gonad scoring shown in this figure was performed by Alexandra Nichitean.

did not display a significant increase in egg-laying defects or low sperm count compared to *mut-16(pk710)* (Fig. 13C and E).

We also quantified gonad size defects using two categories: small gonads and thin distal gonads. For the category of thin distal gonads, none of the RFP+ or RFP- progeny showed a significant trend compared to *mut-16(pk710)* (Fig. 13F). However, we detected a trend that correlated small gonads with the changes in fecundity (Fig. 13D). All the tested generations of RFP+ show a significantly higher proportion of animals with smaller gonads compared to both N2 Bristol and *mut-16(pk710)* (Fig. 13D). Consistent with our prediction, this phenotype was diminished in RFP- generations (Fig. 13D).

3.3.4 Neuronal overexpression of *mut-16* negatively influences germ cell counts transgenerationally

Since the small gonad defect was the category that correlated with the decreased fecundity, we wondered if this was due to a decrease in germ cell counts. Using DAPI-

stained whole worms, we quantified the number of germ cells per gonad arm of RFP+ and RFP- progeny for F1, F3, and F5 generations and compared it to N2 Bristol and *mut-16(pk710)*. Overall, we found that both *mut-16(pk710)* and the *mut-16* neuronal overexpression strains exhibited a significant decrease in germ cell counts compared to wild-type (Fig. 14A). While RFP- progeny showed a stable level of germ cell counts, RFP+ progeny exhibited a progressive decline in germ cell counts between F1 and F5 generations (Fig. 14A). Interestingly, the significant difference in germ cell counts between RFP+ and RFP- progeny arose in the F3 generation, corresponding to the significant differences in the fecundity of those populations (Fig. 14A).

To further characterize the lower germ cell count phenotype of the RFP+ progeny, we examined the germ cell count in the mitotic, transition, and pachytene zones of the germline. Except for the RFP+ F1 generation, germ cell nuclei counts in the transition zone did not show significant changes among the populations (Fig. 14C). In contrast, both the mitotic and pachytene zones of RFP+ progeny exhibited significant decreases in germ cell counts compared to *mut-16* and RFP- progeny (Fig. 14B and D). In the mitotic zone, RFP+ populations showed a significant decrease in germ cell counts compared to both *mut-16(pk710)* and RFP- populations, suggesting that neuronal expression of MUT-16 can regulate the proliferation of the germline stem cell niche. Furthermore, the RFP- progeny were able to rescue their mitotic germ cell counts to *mut-16(pk710)* levels in only one generation without the transgene array (Fig. 14B). This result alludes to the negative consequences of *mut-16* overexpression on germline integrity.

Similarly, germ cell counts in the pachytene zone were significantly lower for all RFP+ progeny compared to *mut-16(pk710)* and RFP- progeny (Fig. 14D). However,

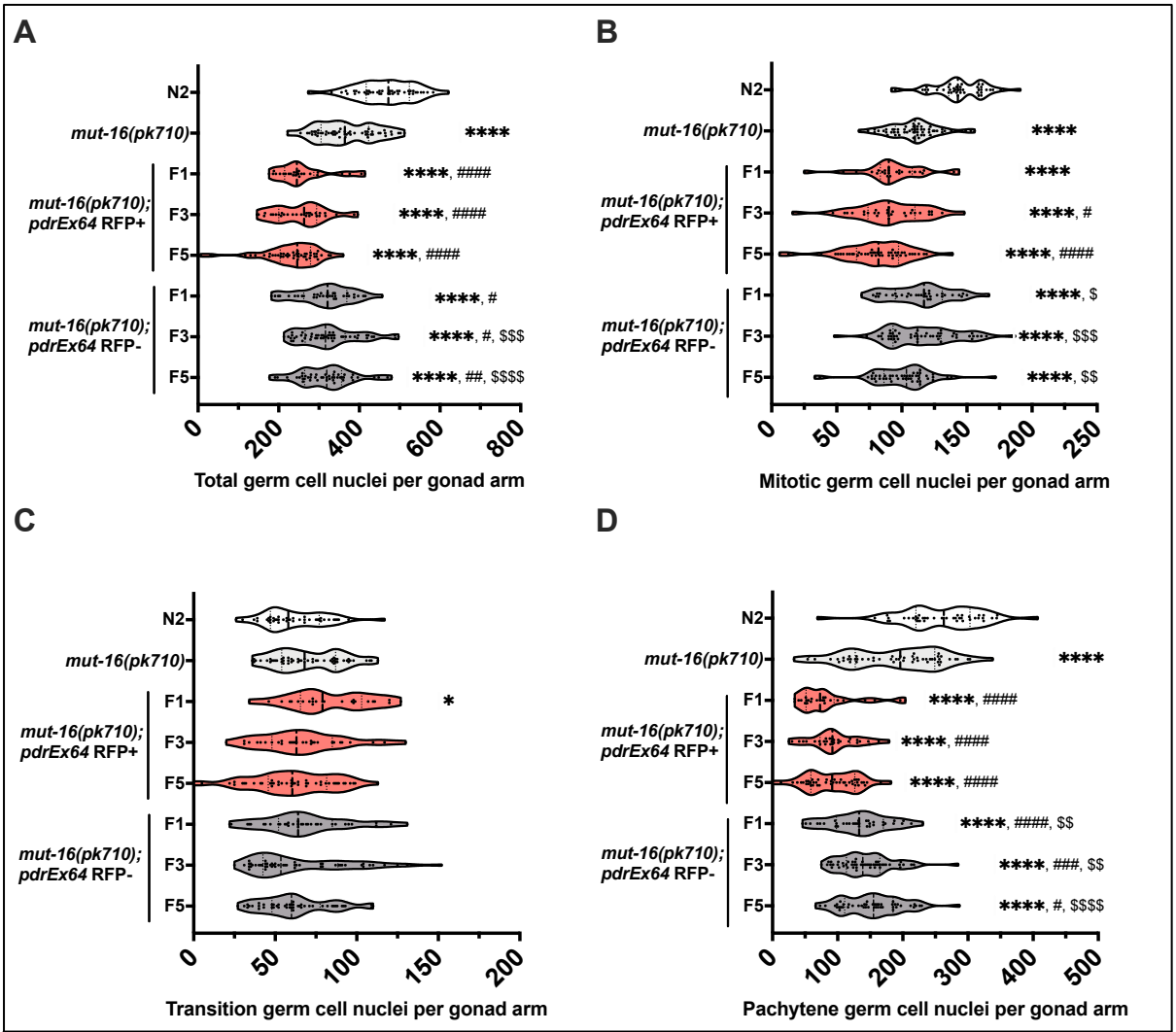


Figure 14: Retention of *mut-16* neuronal overexpression array impacts germ cell counts. Violin plot of the number of germ cell nuclei per gonad arm for N2, *mut-16(pk710), mut-16(pk710); pdrEx64[rab-3*P*::mut-16::gfp],* both RFP+ and RFP-. The germ cell counts shown are for (A) total germ cell nuclei per gonad; (B) mitotic germ cell nuclei per gonad; (C) transition zone germ cell nuclei per gonad arm; and (D) pachytene zone germ cell nuclei per gonad arm. The x-axis denotes the number of germ cell nuclei. The y-axis denotes the genotypes and the different consecutive generations of progeny from a parent carrying the extrachromosomal rescue array. The number of independent trials (N) and number of animals (n) for each genotype and generation are N = 3 trials, n ≥ 14 animals with at least 25 gonad arms examined. One-way ANOVA followed by Tukey's *post hoc* test, *, #, $ $p < 0.05$, **, ##, $$ $p < 0.01$, ***, ###, $$$ $p < 0.001$, ****, ####, $$$$ $p < 0.0001$. * denotes a comparison to N2; # denotes a comparison to *mut-16(pk710),* $ denotes a comparison to RFP+ within the same generation. The germ cell counts shown in this figure were performed by Alexandra Nichitean.

RFP- progeny showed a gradually increasing, intermediate germ cell count compared to *mut-16* and RFP+ progeny, suggesting recovery of the pachytene zone after the loss of the transgene array takes more generations than for the mitotic zone (Fig. 14D). Together, these results indicate that the overexpression of MUT-16 in neurons can negatively impact fecundity and germ cell counts transgenerationally. Overall, we report here the heritable, negative effects of *mut-16* overexpression on fecundity and germline morphology.

3.4 Conclusions

Here, we show that the overexpression of the RNAi component *mut-16* pan-neuronally has adverse effects on germline development and fecundity. A gradual decrease in brood size was observed in the progeny that retained the array, while progeny that lost it maintained a relatively stable brood size over the five generations. This effect was not dependent on having the sensitized *mut-16* background, as a similar trend is observed due to the introduction of the overexpression array into the wild-type background, N2 Bristol. Additionally, we observed germline defects including DTC migration defects, low or no sperm, and abnormal gonad size that were transgenerationally inherited, disproportionately affecting progeny retaining the extrachromosomal array. Additionally, we were able to link the decrease in fecundity in RFP+ progeny to a significant decrease of germ cell counts in both the mitotic and the pachytene zones compared to both *mut-16(pk710)* and RFP- progeny. Overall, we demonstrated a phenomenon whereby RNAi pathways in neurons can influence proper germline development and promote fecundity, and any perturbations can lead to a drastic, heritable, decrease in fertility.

Chapter 4: Discussion

4.1 Model

Here, we show the molecular pathways contributing to increased outcrossing rates following dauer. We show that the increased outcrossing is mostly due to the developmental history of males, with a smaller contribution to this increase via hermaphrodites. When we tested the ability of males to locate mates via pheromones, we observed a significant increase in mate-locating abilities of postdauer males compared to control males (Fig. 10). Additionally, we show that both the TGF-β and RNAi pathways play a role in regulating this phenotype. When either DAF-3 or MUT-16 function is lost in males, we no longer observe the significant increase in PD-H x PD-M cross. We show that functional DAF-3 is also required for hermaphrodites to exhibit increased outcrossing in postdauers. This is possibly through its role in regulating *osm-9* expression in postdauer hermaphrodites. We developed a model in which outcrossing in postdauers is under combinatorial regulation of RNAi, possibly through regulating the expression of G proteins in sensory neurons, and TGF-β pathways.

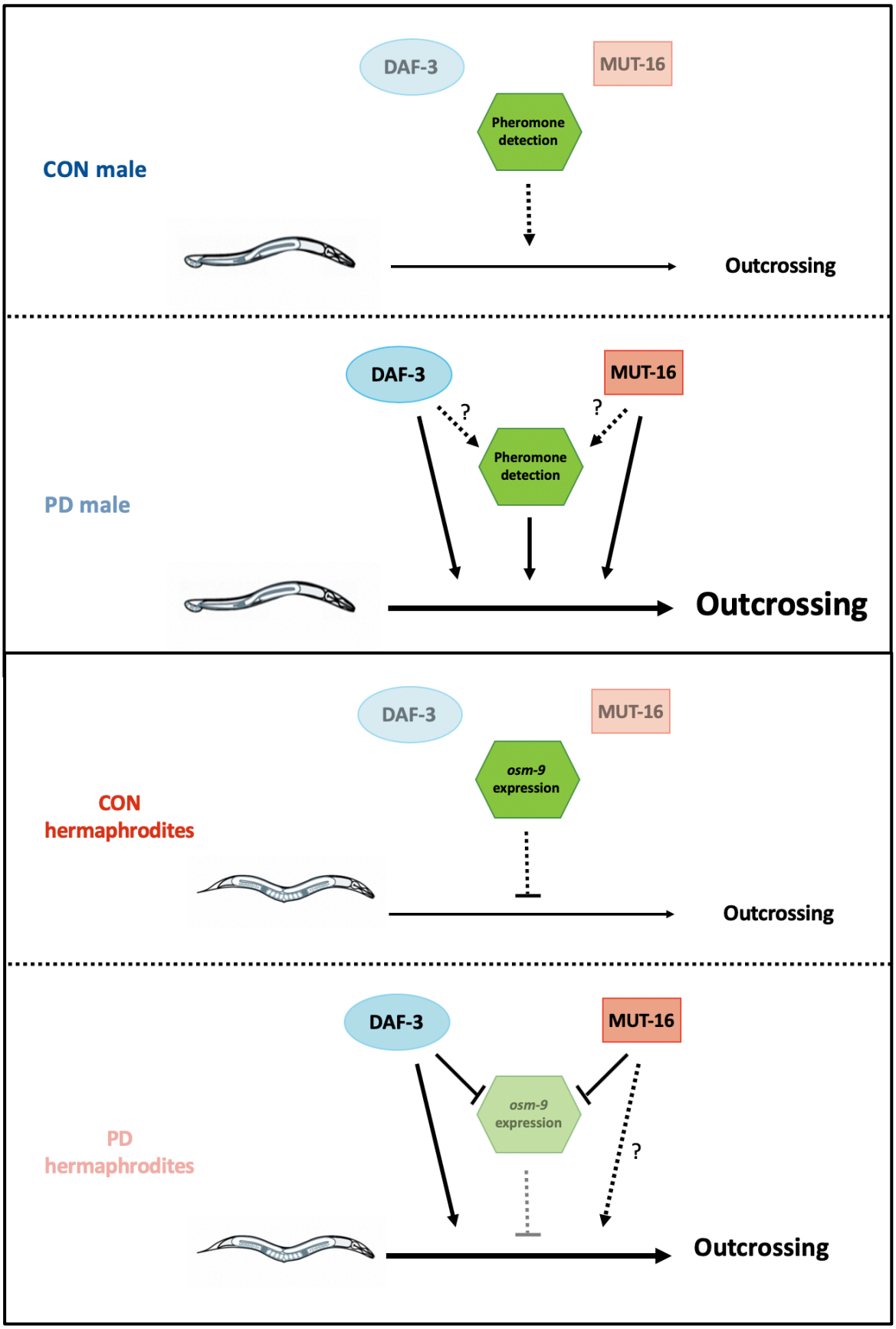

DAF-3
MUT-16
Pheromone detection
CON male
Outcrossing
DAF-3
MUT-16
?
?
Pheromone detection
PD male
Outcrossing
DAF-3
MUT-16
osm-9 expression
CON hermaphrodites
Outcrossing
DAF-3
MUT-16
osm-9 expression
?
PD hermaphrodites
Outcrossing

Figure 15: Model of outcrossing regulation in postdauers. Both DAF-3 and MUT-16 are required for the increased outcrossing phenotype in postdauer males. They might be directly or indirectly influencing their pheromone detection abilities. Hermaphrodites require DAF-3, possibly through its role in regulating *osm-9,* for increased outcrossing following dauer. A solid line denotes a strong influence on outcrossing and a dashed line denotes weak influence. Worm figures adapted from Reuss and C. Schroeder, 2015.

4.2 Postdauer males utilize pheromones to increase the ability to locate mates

Phenotypic plasticity plays a significant role in regulating reproductive modes. Here, we show that the change in reproductive strategies in *C. elegans* following exposure to an adverse environment requires sexually dimorphic changes in behavior. We found that postdauer males are better able to locate hermaphrodites compared to continuously developed males, effectively switching the population from self-fertilization to a mixed mating mode where both self-fertilization and facultative outcrossing are utilized. Furthermore, we found that this increased ability to locate mates is dependent on pheromones. Hence, there could be two non-mutually exclusive mechanisms driving this; upregulation of pheromone sensing genes or suppression of behaviors that antagonize mate finding abilities.

It has been previously shown that postdauer hermaphrodites differentially regulate a large subset of their genes in response to stress (Hall *et al.* 2010; Sims *et al.* 2016; Vidal *et al.* 2018; Ow *et al.* 2018; Bhattacharya *et al.* 2019). Similarly, there could be a differential expression of chemotaxis genes in males such that they become more sensitive to hermaphrodite-produced pheromones and more efficient at locating mates. In order to detect mates, males require functional OSM-9 TRPV channel expression in

the AWA, AWC, and the sex-specific CEM sensory neurons (White *et al.* 2007). In addition to OSM-9, the TRPV channels OCR-1, OCR-2, and the cyclic nucleotide-gated channel TAX-4 have all been shown to be important for males to locate mates, and the loss of these channels eliminated the males' ability to respond to pheromones produced by hermaphrodites (White *et al.* 2007). Thus, these channels might be upregulated in the sensory neurons of postdauer males specifically, making them more sensitive to pheromones. Additionally, males exhibit attraction towards ascr#3 via the sensory neuron ADF, which antagonizes the ADL-mediated ascr#3 avoidance via the RMG inter/motor neuron gap junctions (Fagan *et al.* 2018). An increase in the activity of ADF in postdauer males can additionally contribute to improved mate-locating ability.

Alternatively, this increased attraction can be accomplished by suppressing neuronal circuits that inhibit mate-finding behaviors such as foraging or food-seeking circuits. *C. elegans* males have to balance their drives for reproduction and survival; males will leave bacterial patches that lack mates, risking food loss, in order to find hermaphrodites. While hermaphrodites tend to stay on food sources by reversing backward in response to the edge of bacterial lawns, males will exit faster and more frequently than hermaphrodites in the absence of mates (Barrios 2014; Emmons 2017). Thus, postdauer males might have a lower food-seeking drive, promoting them to seek mates more frequently than control males. This sexual drive is influenced by male tail ray neurons; the output of these neurons results in either local search or dispersal. Local search promotes males to stay on their food source, by reversing at the edges of the bacterial patch, in the event of a recent mate contact (Barrios *et al.* 2008; Barrios 2014). Dispersal, however, promotes food-leaving behavior in the absence of mates through stimulating forward movement in males (Barrios *et al.* 2008; Barrios 2014). Therefore,

there could be a change in activity in the postdauer male ray sensory neurons, promoting exploratory behavior by stimulating food exit and suppressing turns (Barrios *et al.* 2008).

Hermaphrodite-produced "mate-finding" pheromone cues influence males to switch from foraging behavior to mate-locating behavior once they detect these pheromones (Simon and Sternberg 2002; Lipton *et al.* 2004). Similar to this modulation of foraging, postdauer males may be modulating the switch from foraging to mate-finding behavior more efficiently or more frequently than control males, resulting in an increased outcrossing rate. This hypothesis is supported by the recent evidence showing that *C. elegans* hermaphrodites of the Hawaiian wild isolate CB4856 can permanently alter their foraging behavior based on passage through the starvation-induced dauer stage (Pradhan *et al.* 2019). While this change in foraging behavior is not seen in N2 Bristol hermaphrodites, it is unknown how males change their foraging behavior in response to starvation-induced dauer. Males tend to leave their food source to search for mates, a behavior that is suppressed by the presence of hermaphrodites and governed by the nutritional status of the males (Lipton *et al.* 2004). Therefore, there can be a cellular memory of starvation in postdauer males that promotes the suppression of foraging. Additionally, a change in exploratory behavior might contribute to increased outcrossing following exposure to stress. *C. elegans* explores its environment through a random biased walk, occasional turns and reversals that randomly occur throughout its movement, in a biased direction towards favorable chemical cues (Berg and Brown 1972; Pierce-Shimomura *et al.* 1999). This exploratory behavior is regulated by the ASK, ASI, and AWC sensory neurons; a change in the activity of these neurons might increase exploratory behavior in males, and in turn,

increase the probability of locating mates (Gray *et al.* 2005). Finally, a change in the male neuronal circuitry responsible for ascr#3 attraction might underlie the increase in outcrossing rates in postdauers. A change in activity in any of the neurons responsible for ascr#3 attraction or strengthening their connections in males including ADF, ASK, CEM, or the inter/ motor neuron RMG can increase the males' ability to locate mates, thus potentially increasing outcrossing rates.

4.3 The role of *osm-9* in regulating outcrossing frequencies

We showed that change in the outcrossing rate following the dauer diapause is dependent on both RNAi and TGF-β signaling pathways. Due to the role that both pathways play in regulating *osm-9* expression in postdauers, there is a potential contribution of *osm-9* to the increased outcrossing phenotype. One piece of evidence that supports this idea is the effect that the type of hermaphrodite had on outcrossing rate; when *daf-3* hermaphrodites were mated with wild-type males, the trend no longer looked like the wild-type cross from Figure 6, which is to be expected if hermaphrodites played no role in this increased outcrossing phenotype. This alludes to the importance of the developmental experience of hermaphrodites in determining mating frequency after experiencing stress during early development.

While we showed that the change in *osm-9* expression in hermaphrodites has a potential role in regulating outcrossing rates, the role that *osm-9* expression in postdauer males plays in regulating outcrossing is still to be further explored. We observed sex-specific regulation of *osm-9*; unlike in postdauer hermaphrodites, *osm-9* is not downregulated in postdauer males, and no change in ascr#3 avoidance was observed. However, we cannot exclude the hypothesis that postdauer males upregulate

osm-9 expression in ADF, and potentially other neurons contributing to ascr#3 attraction in males such as AWA, AWC, and CEM, thus increasing their ability to locate mates in an *osm-9* dependent manner.

4.4 Overexpression of RNAi components influences fecundity and germline morphology

Previous studies in the Hall lab have shown that RNAi is required for dauer formation that is induced by high pheromone concentrations (Bharadwaj and Hall 2017). While the dauer formation phenotype was rescued when MUT-16 function was restored in any of the sensory neurons known to detect dauer pheromones in the previous studies, we failed to rescue the mating phenotype in postdauers in this study. This can be due to a difference in the neuronal requirements for dauer formation and mating; while dauer formation requires ASI, ADF, ASG, ASJ, ASK, AWA, and AWC neurons, mating requires ADF, AWA, AWC, ASK, and the male-specific CEM neurons (Fielenbach and Antebi 2008). This different neuronal requirement helps execute different behaviors depending on the pheromone concentrations, environmental conditions, developmental stage, and in a sex-specific manner (Park *et al.* 2019). For example, low ascaroside concentrations (pM-fM) attract males, while higher concentrations (nM- μM) result in dauer formation (Srinivasan *et al.* 2008). Additionally, the expression of G protein-coupled receptors (GPCRs) determines the neuronal response to ascarosides; while the ascr#2 receptor DAF-37 promotes dauer formation when expressed in ASI, it promotes repulsion when expressed in ASK (Park *et al.* 2012). Similarly, while dauer formation can be accomplished through restoring the activity of MUT-16 in one of the sensory neurons, there is likely to be communication

and a fine balance between multiple neurons regulating outcrossing. Thus, restoring the activity of MUT-16 in single neurons was apparently not sufficient to restore outcrossing to wild-type levels. Outcrossing may have been restored in these lines, but it was masked by *mut-16(pk710)* sterility.

Overexpression of *mut-16* pan-neuronally not only failed to restore proper levels of outcrossing, it completely abolished mating and negatively influenced the gonad morphology and fecundity. This is likely due to the role of MUT-16 in siRNA amplification (Zhang *et al.* 2011; Phillips *et al.* 2012; Uebel *et al.* 2018). There is evidence that dsRNA can be transported from the soma to the germline and regulate gene expression transgenerationally (Gu *et al.* 2012; Ashe *et al.* 2012; Devanapally *et al.* 2015; Spracklin *et al.* 2017; Kalinava *et al.* 2018). Thus, it is possible that the overexpression of *mut-16* leads to accumulation of dsRNAs in neurons that are then transported to the germline. These dsRNAs may then contribute to aberrant gene expression and chromatin modifications in the germline, triggering epigenetically heritable germline defects. This could be possible through overexpression of DTC migration genes, or downregulation of the Notch signaling pathway that maintains the mitotic cell niche (Wong and Schwarzbauer 2012; Kimble 2014; Kipreos and Van Den Heuvel 2019). This can be further investigated using chromatin immunoprecipitation (ChIP) or RNA sequencing (RNA-seq) to examine the epigenetic changes between N2, *mut-16,* and the *mut-16* pan-neuronal rescues over generations. When hermaphrodites inherit extrachromosomal arrays overexpressing *mut-16,* gonad morphology is significantly impacted; a significant number of animals are observed to have a thin gonad in addition to a low number of germ cells. This decrease in germ cells, specifically in the mitotic and pachytene zones, suggests MUT-16 dependent suppression of

germline proliferation, resulting in a significant decrease in germ cell counts in the presence of the extrachromosomal arrays. This effect on fecundity and germline is reversible, as losing the array over generations results in a restoration of germ cell counts to levels expected for that genetic background (*mut-16)*. This research elucidated the genetic mechanism driving changes in reproductive strategies in response to environmental changes. It ultimately adds to the growing body of examined mechanisms of different reproductive strategies and how environmental changes can significantly impact the mating strategies and outcomes. This is accomplished by linking changes at the molecular level to changes in population dynamics that ultimately drive genetic diversity.

4.5 Reproductive plasticity as an evolutionary driver of adaptation

It is important to view the findings of this project in the context of evolution, as these changes in reproductive strategies may be driving adaptation. The changes in outcrossing rates and differential gene expression patterns are all likely to be mechanisms promoting populations to adapt to their environment by generating genetic diversity. Here, we show that environmental stress during early development influences the switch of reproductive systems from primarily self-fertilization to a mixed mating system of both self-fertilization and facultative outcrossing. This change has a significant evolutionary implication; by allowing increased mating after stress, increased genetic diversity is promoted by shuffling alleles in the population. This genetic diversity will make populations likely to be more adaptive to future stressors. Additionally, this will provide primarily selfing populations of the benefits of outcrossing, such as decreasing inbreeding depression and preventing the accumulation of deleterious

mutations, while preserving the benefits of self-fertilization such as the higher propagation efficiency and decreased sexual conflict.

C. elegans is mostly thought of as the powerful genetic model that is used in laboratories in its continuously developing form, considering environmental stress a temporary and infrequent event. However, in its natural habitat, *C. elegans* is found mostly as its migratory dauer form, exiting when it finds favorable conditions (food availability, suitable temperatures, areas with low population density) (Barrière and Félix 2005, 2007; Frézal and Félix 2015). Thus, it is likely that hermaphrodites outcross more in nature compared to what we observe under laboratory conditions. This has been determined to be true by studies looking at the strength of linkage disequilibrium groups in N2 compared to recent wild isolates; N2 has very strong linkage groups, extending to chromosomes, compared to the recent wild isolates examined which suggests that N2 has lower outcrossing rates compared to those wild isolates (Cutter 2006). Males are more efficient at entering the dauer diapause, better able to survive the dauer diapause, actively attracted to dauer pheromones, and have a higher affinity to mates following dauer (Ailion and Thomas 2000; Morran *et al.* 2009a; Ludewig and Schroeder 2013). All of these characteristics make males a perfect tool for driving genetic diversity after dauer exposure. In natural habitats, this change in reproductive strategies in response to stress provides a mechanism to increase the genetic diversity of the population.

4.6 Future directions

Certain questions that need to be addressed by future researchers include the mechanisms by which males change their ability to locate mates. First, what changes, at

the molecular levels, are inducing males to outcross more following dauer diapause? To address this, the first step should be to conduct an RNA-seq experiment on both CON and PD males to examine all the genes that are differentially expressed due to passage through dauer. Due to the rarity of males in N2 Bristol, this experiment can be conducted in a *him-5* genetic background or by using a different wild isolate with a high male incidence such as the Hawaiian wild isolate CB4856. Hermaphrodites can be eliminated from one of the experimental populations using a variety of tools, possibly through an inducible *peel-1* gene that can be induced under a hermaphrodite-specific promoter such as *vit-2*, thus ensuring its expression in hermaphrodites only (Yi and Zarkower 1999). Another possible tool to use is the Auxin Inducible Degron, a protein degradation mechanism initiated once worms are supplemented with the hormone auxin, to induce toxicity in hermaphrodites (Zhang *et al.* 2015). Alternatively, males can be sorted out of the population using a fluorescent marker, creating a hermaphrodite-only sample and a male-only sample, that can be used for RNA-seq.

The next question to be addressed is what neurons are required for increased outcrossing in postdauer males? While neuronal ablation of certain neurons, such as the ADF neuron, eliminates attraction to mates, it would be interesting to unveil other neurons that either act in the mate-finding circuit in postdauers exclusively, or are common in both CON and PD conditions (Fagan *et al.* 2018). Calcium imaging coupled with microfluidics can be utilized to examine the neuronal response to pheromones produced by mates (either male or hermaphrodite response) in real-time (MacOsko *et al.* 2009; Kim *et al.* 2009; Borne *et al.* 2017). The neuronal response of CON and PD animals will be informative in constructing a circuit determining mate preference in response to different environmental or developmental events.

Another question that should be addressed is the mechanism by which *mut-16* overexpression decreases fecundity and alters gonad morphology. It would be interesting to investigate other RNAi components to test whether they have similar effects on the germline. This can be accomplished by creating extrachromosomal rescue constructs similar to that of *mut-16,* driving the expression of other *Mutator* proteins pan-neuronally, and examining their effect on fecundity transgenerationally. If there are any significant differences over generations in brood size, the next step would be examining their gonad morphology to assess any potential defects. Additionally, to investigate whether this effect is due to the transportation of dsRNAs into the germline, the same transgenerational brood size experiments done in chapter 3 can be repeated in a *sid-1* background. SID-1 is a dsRNA channel that is required for systemic RNAi and the transport of dsRNAs between different tissue types (Shih and Hunter 2011). If this effect is dependent on SID-1, we expect the difference in brood size and gonad morphology defects between RFP+ and RFP- to be abolished.

Chapter 5: Appendix

5.1 List of strains used in this study

GR1311 [*daf-3(mgDf90)* X]

GR1823 [*mut-16(mg461)* I]

NL 1810[*mut-16(pk710)* I]

YY158 [*nrde-3(gg66)* X]

SH236 [*mut-16(pk710); pdrEx64(rab-3*p*::mut-16::gfp)*]

SH378 [*pdrEx103(rab-3*p::*mut-16::gfp;coel::dsRed)*]

SH360 [*mut-16(pk710); pdrIs12(rab-3*p*::mut-16::gfp;coel::dsRed)*]

SH286 [*mut-16(pk710); pdrEx69(gpa-4*p*::mut-16::gfp)*]

SH398 [*mut-16(pk710); pdrIs16(gpa-4*p*::mut-16::gfp;coel::dsRed)*

SH288 [*mut-16(pk710); pdrEx71(sre-1*p*::mut-16::gfp)*]

SH277 [*mut-16(pk710); pdrEx73(srh-142*p*::mut-16::gfp)*]

UR926 [*him-5(e1490) V*]

UR1217 [*unc-54(e190) I; him-5(e1490) V*]

UR1218 [*unc-54(e190) I; daf-22(m130) II; him-5(e1490) V*]

EG4322 [*ttTi5605 II; unc-119(ed9) III*]

5.2 Mating assays sample sizes

Mating assays described in 2.2.2 were all set up with six hermaphrodites and six males, with a few exceptions listed below. For simplicity, CON-H x CON-H will be labeled A, CON-H x PD-M is B, PD-H x CON-M is C, and PD-H x PD-H is D.

1. One N2 biological replicate was set up with 12 animals of each sex for all crosses.
2. One *daf-3* biological replicate was set up with 14 animals of each sex for all crosses.
3. One *mut-16* biological replicate was set up with 5x5 for B and 4x4 for D. Another biological replicate was set up with 5x5 for B and D.
4. One *mut-16* pan-neuronal rescue biological replicate was set up with 6x4 for B and 5x5 for D.
5. Three *mut-16* ADL rescue biological replicates were set up with 3x3 for A and B. Two trials were set up with 3x3 for C. One trial was set up with 4x4 for D.
6. One *mut-16* ASI rescue biological replicate was set up with 4x4 for B and 3x3 for C.
7. One *mut-16* ADF rescue biological replicate was set up with 4x4 for B and D.

5.3 Male frequencies for the strains used in mating assays

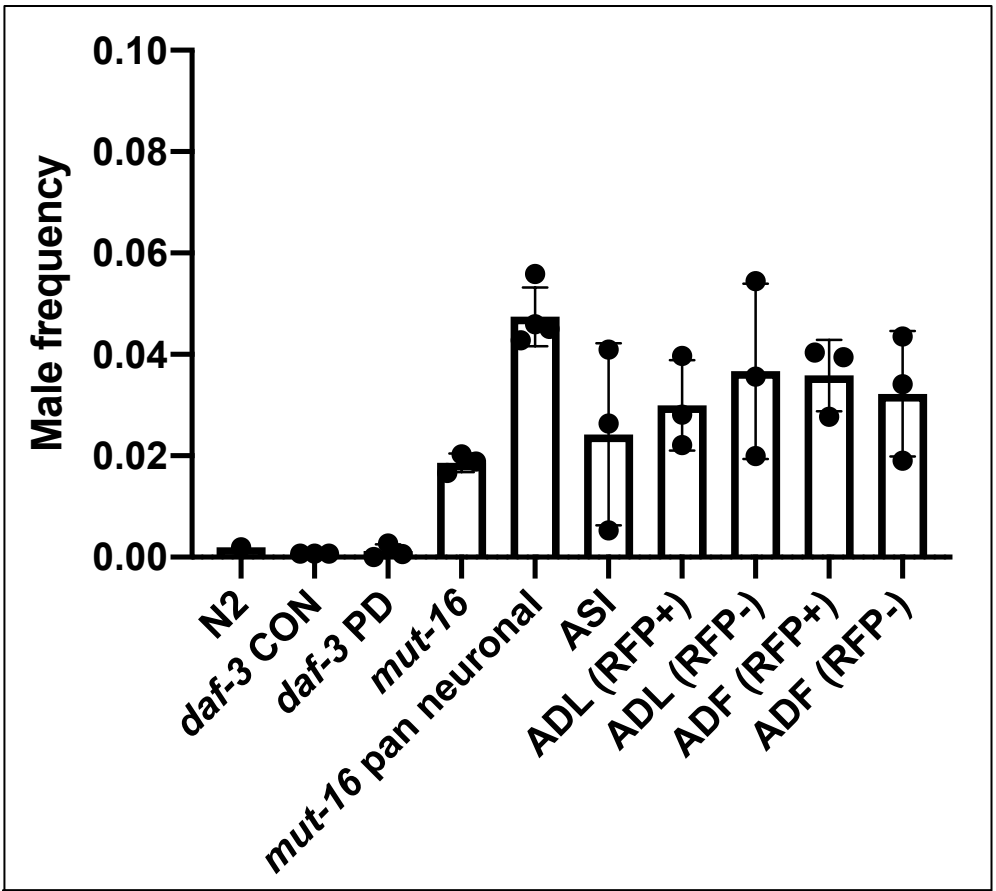


Figure S1: *mut-16* strains exhibit increased male incidence. Mean male frequency ± SEM. The value for N2 male frequency is reported in the literature. The number of independent trials (N) and number of animals (n) is: N ≥ 3 trials, n ≥ 18 animals.

While N2 Bristol has a low percentage of males in the population due to X-chromosome nondisjunction that has been reported previously, we wanted to account for the exact proportion of males that are not generated by outcrossing in the other tested strains (Ketting *et al.* 1999; Vastenhouw *et al.* 2003). To do so, we conducted brood size assays using three biological replicates of each genotype, with 6 hermaphrodites tested for each trial. The only exception is the *mut-16* pan-neuronal rescue, where 4 biological replicates were used, one being an average of three technical

replicates (Fig. S1). Since *daf-3(mgDf90)* is a dauer deficient strain, populations were grown on 100mM plates seeded with concentrated bacteria (20x OP50) at 25°C for a week to induce dauer formation. After the plates are starved and dauers are observed, an SDS wash is used to separate dauers from n0n-dauers. There was no significant difference between CON and PD male frequencies, thus CON male frequency values were subtracted from either condition. The male frequency estimates due to X-chromosome nondisjunction were subtracted from the mating assays' male frequencies, matching conditions when possible.

5.4 1M glycerol and M13 avoidance indices

Worms were tested for avoidance behavior in response to 100 mM ascr#3, with

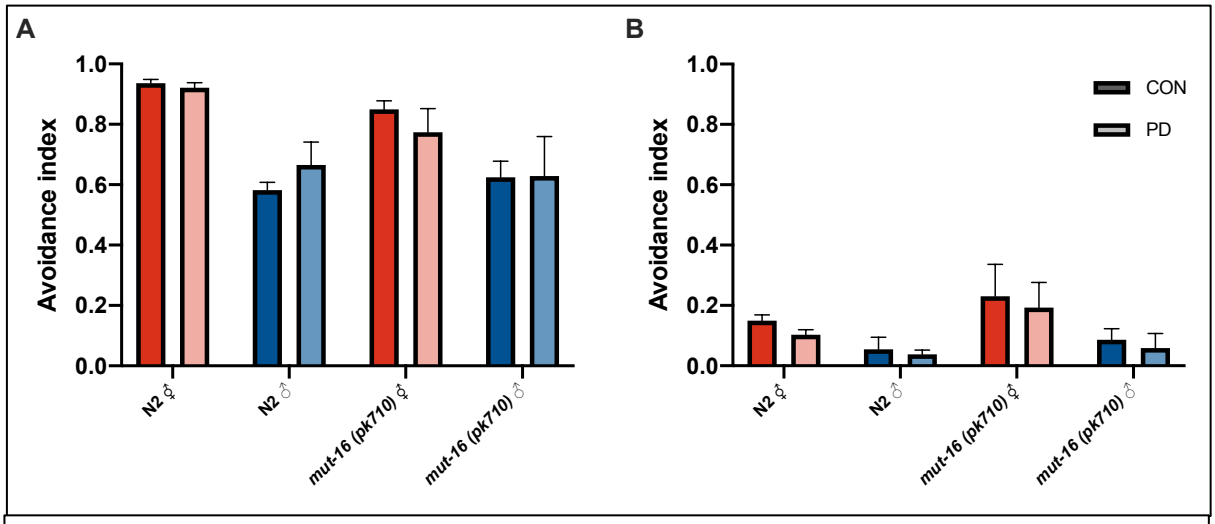


Figure S2: 1M glycerol and M13 avoidance indices. Avoidance index ± SEM for N2 Bristol and *mut-16(pk710)* hermaphrodites and males in response to (A) the positive control 1M glycerol and (B) the negative control M13. The number of independent trials (N) and number of animals (n) for A: N ≥ 3 trials, n ≥ 76, B: N ≥ 3 trials, n ≥ 76 animals. Multiple *t*-tests.

1M glycerol being used as the positive control and M13 as the negative control. The method is described in detail in section 2.2.4. The proportion of worms responding to ascr#3 is normalized by subtracting the proportion of worms responding to the positive control (M13) as previously described (Hilliard *et al.* 2002; Jang *et al.* 2012; Sims *et al.* 2016). There were no significant differences between CON and PD in any of the comparisons. The number of biological replicates for both 1M glycerol and M13 avoidance and the total number of animals tested can be found in Table 4.

Response to 1M glycerol				
Genotype	**Sex**	**Condition**	**Biological replicates (N)**	**Sample size (n)**
N2	Hermaphrodite	CON	27	601
		PD	25	575
	Male	CON	3	93
		PD	5	152
mut-16(pk710)	Hermaphrodite	CON	5	154
		PD	4	116
	Male	CON	6	135
		PD	4	76
Response to M13				
Genotype	**Sex**	**Condition**	**Biological replicates (N)**	**Sample size (n)**
N2	Hermaphrodite	CON	27	612
		PD	25	569
	Male	CON	3	94
		PD	5	155
mut-16(pk710)	Hermaphrodite	CON	5	153
		PD	4	117
	Male	CON	6	137
		PD	4	76

Table 4. Number of biological replicates and sample sizes for pheromone avoidance assay controls. Biological replicates represent assays set up independently. Sample size represents the total number of animals tested for a specific genotype.

5.5 Neuronal overexpression of *mut-16* impacts gonad morphology

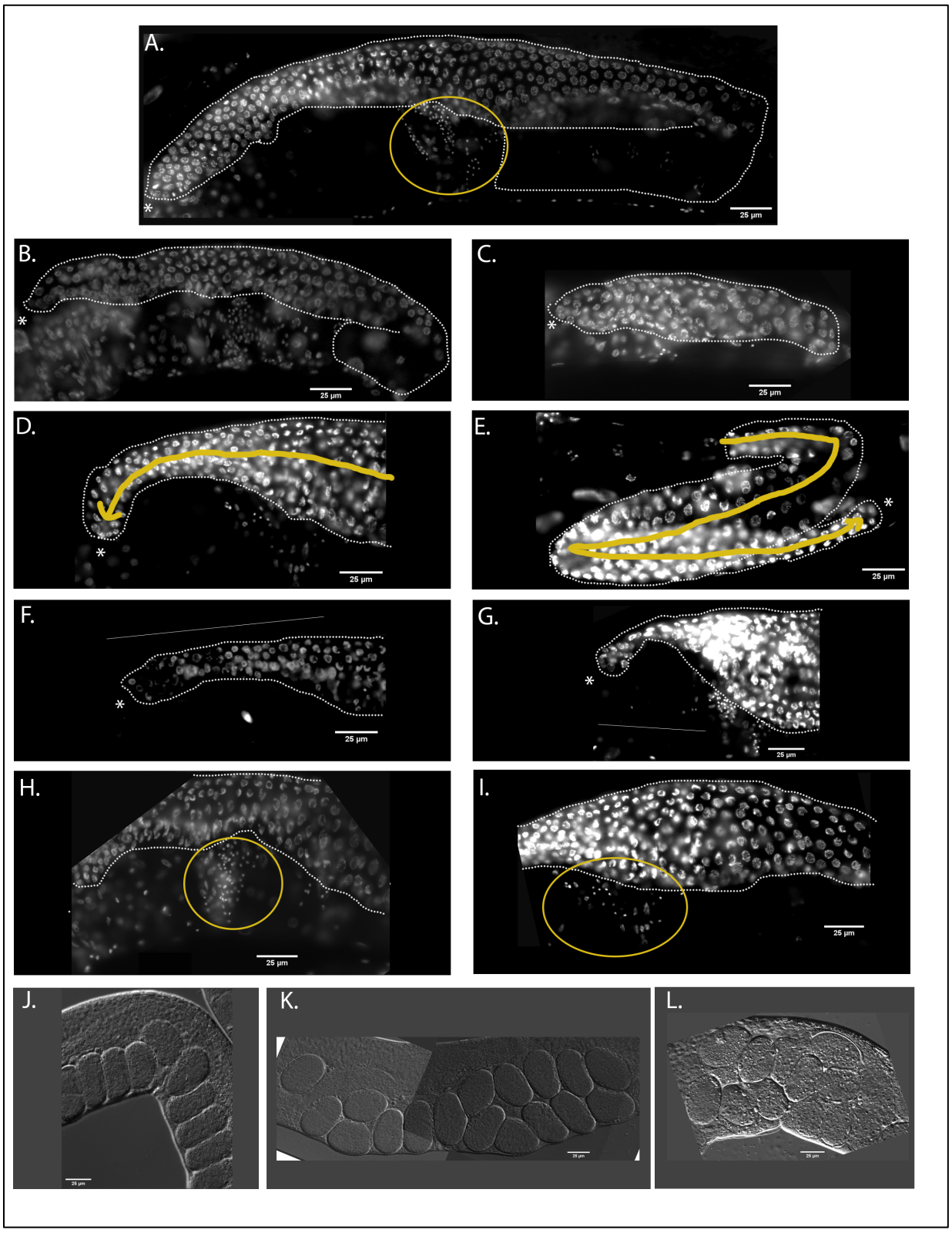


Figure S3: Neuronal overexpression of *mut-16* impacts gonad morphology.
Overexpression of *mut-16(pk710)* in the neurons in a *mut-16* mutant background correlates with a variety of gonad defects. A.) A representative wild type gonad. B-C) A mild (B) and severe (C) example of a smaller gonad size compared to wild type. D-E) A mild (D) and severe (E) example of DTC migration defects found in the animal lines carrying mutations, yellow arrow indicates the potential path the DTC might have taken. To note all images are arranged with the DTC and the location of the vulva facing the left side, however in E.) the DTC has done an additional turn and is facing the posterior end of the animal away from the location of the vulva. F-G) Examples of a thin (F) and an extremely thin distal gonad size compared to wildtype, the line indicates the areas that visually appear thinner compared to the distal gonad of wild type. H-I) An example of low (H) to extremely low (I) sperm number seen in the mutant animals compared to wild type, the yellow circle indicates the spermatheca. J-L) The uterus with eggs seen in wild type (J), *mut-16(pk710)* animals with a mild (K) and a severe (L) example of embryo retention. Scale bar is 25µm.

5.6 Neuronal overexpression of *mut-16* impacts adult germline

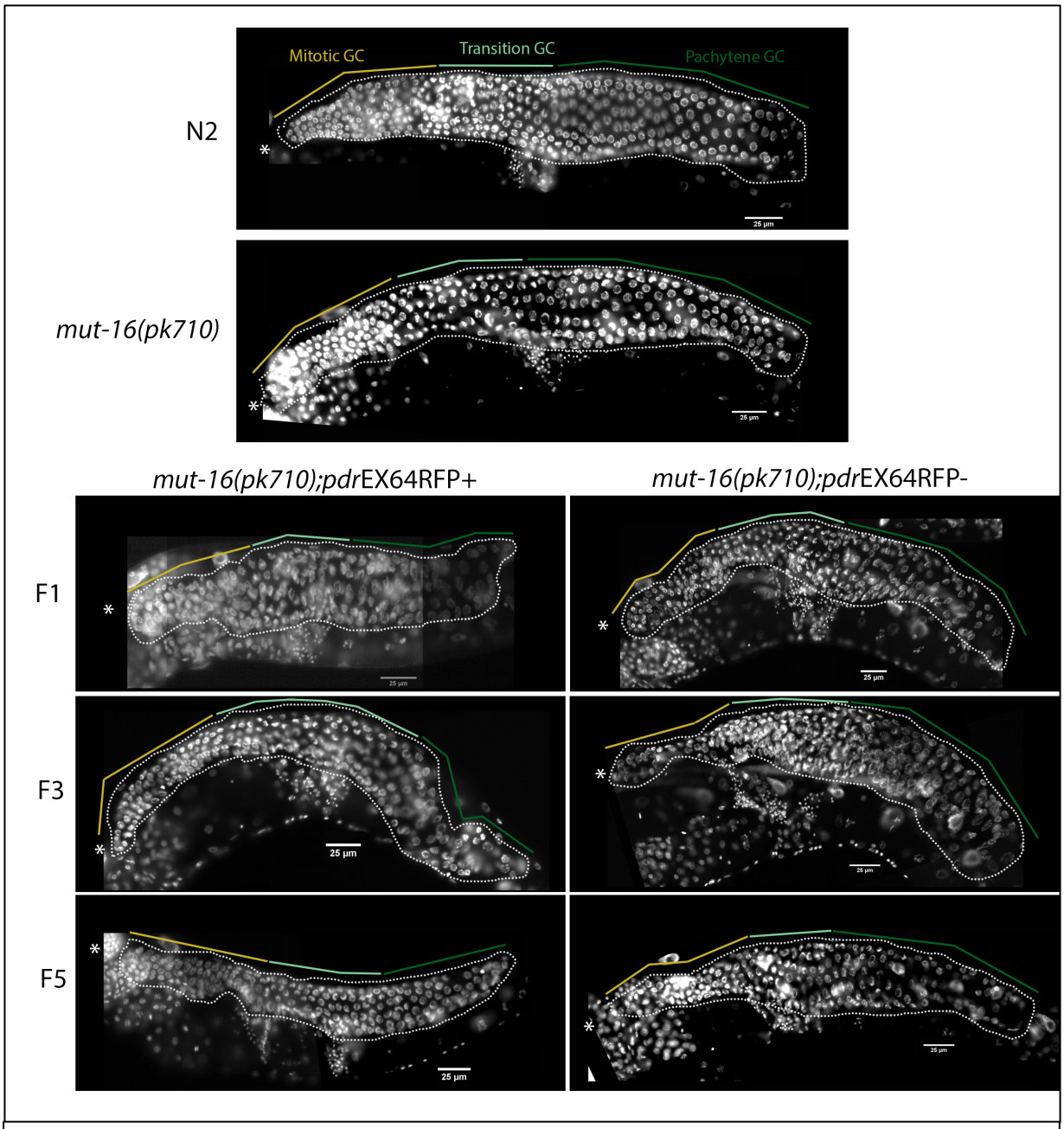


Figure S4: Neuronal overexpression of *mut-16* impacts adult germline.
Representative images of DAPI stained whole mount gonads of N2, *mut-16(pk710)* and three generations of the *mut-16(pk710);pdr*Ex64RFP+ and RFP- animals. The yellow, light green and dark green lines indicates germ cell nuclei undergoing mitotic, transition, and meiosis pachytene, respectively. The white dotted line delimits the mitotic, and leptotene, zygotene, and pachytene part of meiosis of young adult animals. Asterisk symbol indicates the DTC. Scale bar is 25µm.

5.7 The role of NRDE-3 argonaute in *osm-9* regulation

We have previously shown that NRDE-3 is required for *osm-9* downregulation in postdauer hermaphrodites; functional NRDE-3 is required to recruit DAF-3 SMAD and the chromatin remodeler ZFP-1 to the *osm-9* locus, downregulating it in postdauer hermaphrodites (Sims *et al.* 2016). However, the mechanism by which NRDE-3 regulates *osm-9* and establishes tissue-specific repression has not yet been explored. Specifically, the question we wanted to address is whether NRDE-3 targets *osm-9* directly or indirectly in ADL neurons. Additionally, the mechanism by which the RNAi machinery is recruited to endogenous loci has not yet been elucidated. To explore this mechanism and to address the question of whether *osm-9* is a direct or indirect target of NRDE-3, we created the strain, *srh-220*p::*c-Myc::nrde-3,* that expresses epitope-tagged NRDE-3 specifically in ADL neurons.

5.7.1 Generation of mosSCI targeting vector

In order to create a mosSCI line, a targeting vector containing the desired insert, a positive selection marker, and regions homologous to the insert site has to be generated. To assemble the targeting vector, the NEB Gibson assembly cloning kit (catalog# E5510S) was used (Fig. S5) (Gibson *et al.* 2009, 2010). The AflII restriction endonuclease was used to linearize the pCFJ151 vector, a mosSCI vector containing the *C. briggsae unc-119* rescue gene in addition to sequences homologous to the flanking region of the Mos1 transposon, ttTi5605. The linearized vector was then incubated with two PCR products with overlapping ends, the *srh-220* promoter, and the *nrde-3* coding sequence tagged with a c-Myc tag at the 5' end of the gene. The assembled targeting

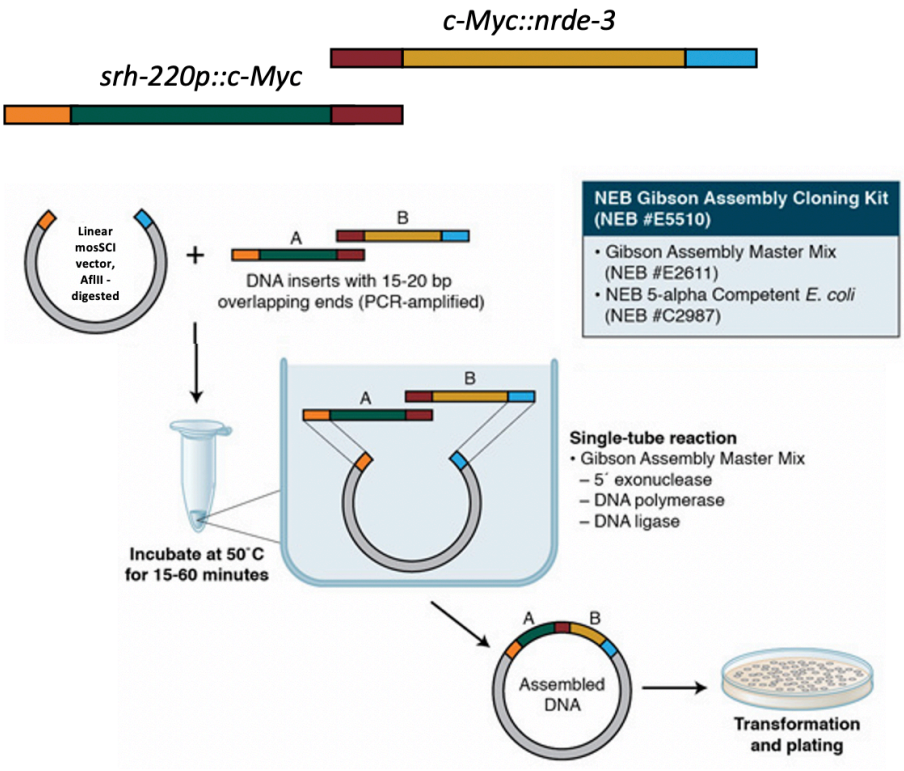


Figure S5: Gibson assembly protocol. The linearized vector and PCR products are incubated in a single-tube reaction to create the assembled DNA plasmid. Figure adapted from NEB.

vector is then transformed into NEB 5-alpha Competent *E. coli* and candidate colonies were screened for the inserts, then the vector was sequenced to ensure no mutations were introduced during the process. The sequenced vector was used for mosSCI.

5.7.2 Strain generation via mosSCI

We used mos1- mediated single-copy insertion (mosSCI) in order to generate the ADL-specific *nrde-3* rescue. This method allows for the genomic integration of genes of interest, allowing for near-endogenous levels of expression (Frøkjær-Jensen *et al.* 2008, 2012). When worms are injected with the mosSCI injection mix, there is a mixture of

worms containing extrachromosomal arrays and worms with successful integration events. In order to select only worms with the integrated *nrde-3* rescue, there has to be negative selection against animals carrying extrachromosomal arrays, that can be lost over generations.

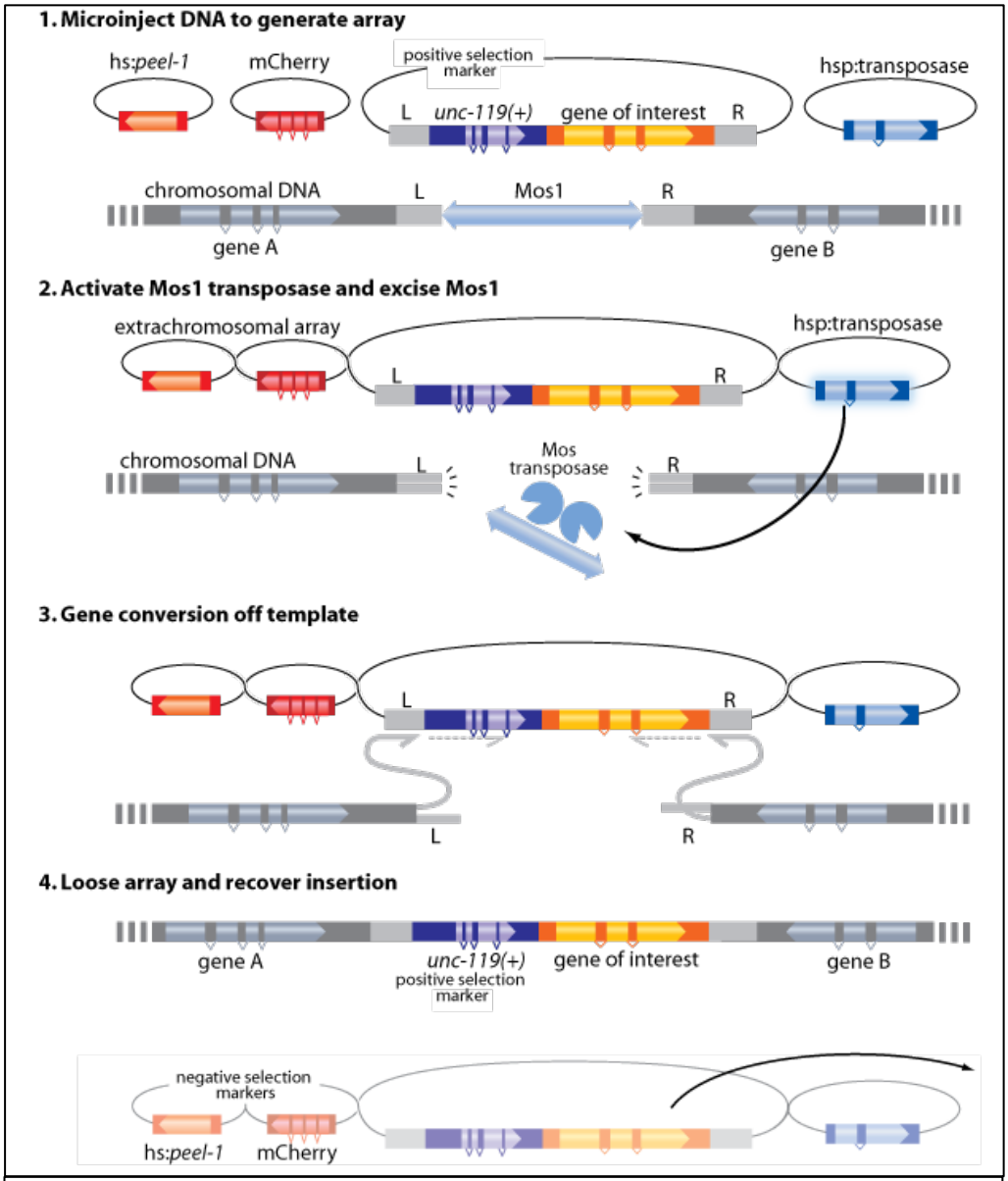


Figure S6: MosSCI protocol. MosSCI injection mix is injected into *unc-119* worms, introducing a rescue transgene to visually select for successful injections. Figure from Frøkjær-Jensen *et al.* 2008.

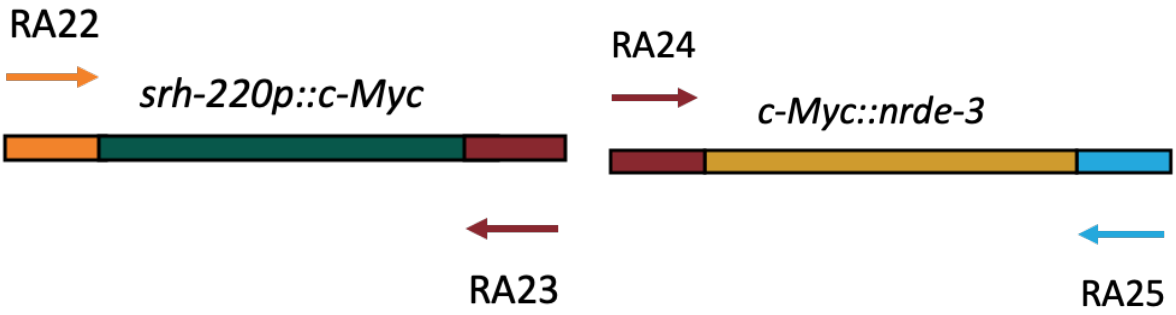
The injection strain for mosSCI was *unc-119(ed9),* a strain with a severe uncoordinated movement and a coiled body phenotype. The targeting vector contained a wild-type *unc-119* gene, which rescued the defective phenotypes in successfully injected worms, allowing for convenient visual selection. In addition to the targeting vector, visual selection markers included the fluorescent extrachromosomal array markers driving the expression of the red fluorescent protein, *mCherry,* in the nervous system, the pharynx, and the body wall muscles (Fig S6) (Frøkjær-Jensen *et al.* 2008). PEEL-1*,* a toxin expressed in spermatocytes whose antidote, ZEEL-1, expressed in embryos, is a system that evolved to decrease genetic incompatibility, which can be leveraged to induce toxicity if expressed alone in adults (Seidel *et al.* 2011). A heat-shock induced *peel-1* was used as a negative selection marker to select for integration events, eliminating any animals retaining extrachromosomal arrays. The last component in the injection mix was the heat-shock induced Mos1 transposase, which will excise Mos1 from its genomic location, and homologous recombination will repair this site using the targeting vector. DNA ladder was added to the injection mix to increase the efficiency of injections. After young adult *unc-119* hermaphrodites were microinjected in the distal gonad arms with the injection mix, they were allowed to propagate for a week at 25°C until the plates were depleted of food. The worms were then heat-shocked at 34°C for two hours and screened for insertions after 5-6 hours. We had the most success by incubating the heat-shocked plates at 15°C for a week then screening for non-fluorescent, non-*unc* worms. PCR was used to verify that the full-length gene had been integrated properly. Worms that experienced successful injections were allowed to self so that the insertion would be homozygous.

5.7.3 *osm-9* regulation by NRDE-3, hypotheses and experimental design

To address the question of whether *osm-9* is a direct or indirect target of NRDE-3, we created the strain, *srh-220*p*::c-Myc::nrde-3,* that expresses epitope-tagged NRDE-3 specifically in ADL neurons. One of the benefits of using mosSCI to generate this rescue transgenes was having an integrated copy of the gene with an expression level close to endogenous levels. Alternatively, extrachromosomal arrays introduce hundreds of copies of the rescue transgene with varying, sometimes very low, transmission rates (Frøkjær-Jensen *et al.* 2012).

Future experiments will use this strain to perform a chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) to investigate whether *osm-9* are enriched in the NRDE-3 pulldowns from ADL. The two potential outcomes are either an enrichment of *osm-9* sequences bound to NRDE-3, or no enrichment of that locus. The first outcome will be evidence for NRDE-3 regulating *osm-9* directly. The second outcome will be evidence for either an indirect regulation of *osm-9* expression by NRDE-3 or regulation of downstream proteins that contribute to ascr#3 avoidance. To distinguish between these two explanations, the expression levels of *osm-9* will be measured via qPCR in both wild-type hermaphrodites and *nrde-3* mutant hermaphrodites. If we observe misregulation of *osm-9* expression in *nrde-3* mutants, that will allude to an indirect role of NRDE-3 in *osm-9* regulation.

5.7.4 MosSCI primers



Primer sequences used to amplify genomic *srh-220* promoter sequences and *nrde-3* coding sequences. The sequence corresponding to the c-Myc tag is highlighted in red.

Table 5: MosSCI primers

Primer name	Primer type	Product	Sequence
RA22	Fw	*srh-220*p with a mosSCI vector overhang at the 5' end and a *c-Myc* overhang at the 3' end	ggtgagctctggtaccctctagtcaag gccgaaagcgttaaccgtgcaaataaccgataa
RA23	Rev		caagtcttcctcggagattagcttttgttc CATacttgagtttggaccgaaaagccagct
RA24	Fw	*nrde-3* with a *c-Myc* overhang at the 5' end and mosSCI vector overhang and at the 3' end	gaacaaaagctaatctccgaggaagacttg GATCTCCTAGACAAAGTAATGGGTGAGATG
RA25	Rev		ggcccgggctacgtaatacgactcactt aaccctggtaaaccatgaatttattggttggt

5.8 Statistical analysis of mating assays

Table 6: Two-way ANOVA table for mating assays

N2 cross statistical analysis					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.0008139	1	0.0008139	F (1, 30) = 0.06742	0.7969
Hermaphrodite type	0.01890	1	0.01890	F (1, 30) = 1.566	0.2205
Male type	0.1448	1	0.1448	F (1, 30) = 11.99	0.0016
Residual	0.3622	30	0.01207		
***daf-3(mgDf90)* cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.008921	1	0.008921	F (1, 28) = 0.8544	0.3632
Hermaphrodite type	0.01328	1	0.01328	F (1, 28) = 1.272	0.2690
Male type	0.04565	1	0.04565	F (1, 28) = 4.372	0.0457
Residual	0.2924	28	0.01044		
N2 hermaphrodite x *daf-3(mgDf90)* male cross statistical analysis					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	2.853e-005	1	2.853e-005	F (1, 11) = 0.003326	0.9550
Hermaphrodite type	0.03934	1	0.03934	F (1, 11) = 4.587	0.0554
Male type	0.07074	1	0.07074	F (1, 11) = 8.248	0.0152
Residual	0.09434	11	0.008576		
***daf-3(mgDf90)* hermaphrodite x N2 male cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.01326	1	0.01326	F (1, 12) = 1.608	0.2288
Hermaphrodite type	1.777e-005	1	1.777e-005	F (1, 12) = 0.002155	0.9637
Male type	0.05235	1	0.05235	F (1, 12) = 6.349	0.0269
Residual	0.09895	12	0.008246		

***mut-16(pk710)* cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.0004835	1	0.0004835	F (1, 8) = 0.1002	0.7597
Hermaphrodite type	2.298e-005	1	2.298e-005	F (1, 8) = 0.004760	0.9467
Male type	0.007782	1	0.007782	F (1, 8) = 1.612	0.2399
Residual	0.03862	8	0.004827		
***mut-16(pk710)* pan neuronal rescue cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.002194	1	0.002194	F (1, 8) = 9.410	0.0154
Hermaphrodite type	0.0002675	1	0.0002675	F (1, 8) = 1.147	0.3154
Male type	1.499e-006	1	1.499e-006	F (1, 8) = 0.006430	0.9381
Residual	0.001865	8	0.0002331		
***mut-16(pk710)* ASI rescue cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	1.935e-005	1	1.935e-005	F (1, 8) = 0.04557	0.8363
Hermaphrodite type	1.181e-007	1	1.181e-007	F (1, 8) = 0.0002781	0.9871
Male type	0.0002993	1	0.0002993	F (1, 8) = 0.7047	0.4256
Residual	0.003397	8	0.0004246		
***mut-16(pk710)* ADL rescue cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.0002116	1	0.0002116	F (1, 10) = 0.2401	0.6347
Hermaphrodite type	0.001104	1	0.001104	F (1, 10) = 1.253	0.2891
Male type	0.0002382	1	0.0002382	F (1, 10) = 0.2704	0.6144
Residual	0.008812	10	0.0008812		

***mut-16(pk710)* ADF rescue cross statistical analysis**					
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	***P* value**
Interaction	0.002252	1	0.002252	F (1, 16) = 0.4762	0.5001
Hermaphrodite type	0.02961	1	0.02961	F (1, 16) = 6.261	0.0236
Male type	0.008633	1	0.008633	F (1, 16) = 1.826	0.1955
Residual	0.07567	16	0.004729		

5.9 Statistical analysis of *osm-9* expression

Table 7: Multiple *t*-tests table for *osm-9* expression

***osm-9* expression statistical analysis**									
Multiple *t*-test table	Significant?	*P* value	Mean of CON	Mean of PD	Difference	SE of difference	t ratio	df	Adjusted *P* Value	Significant?
Hermaphrodite	Yes	0.000134	73.00	31.60	41.40	7.513	5.510	12.00	0.000268	Yes
Male	No	0.628988	80.15	83.88	-3.725	7.513	0.4958	12.00	0.628988	No

5.10 Statistical analysis of mate preference assays

Table 8: Unpaired *t*-test tables for mate preference assays

Mate preference assay statistical analysis	
Unpaired *t*-test table of CON vs PD males in response to immobilized hermaphrodites	
P value	0.0259
P value summary	*
Significantly different ($P < 0.05$)?	Yes
One- or two-tailed *P* value?	Two-tailed
t, df	t=2.330, df=34
How big is the difference?	
Mean of CON (A)	0.1544
Mean of PD (B)	0.3594
Difference between means (B - A) ± SEM	0.2051 ± 0.08799
95% confidence interval	0.02623 to 0.3839
R squared (eta squared)	0.1377
F test to compare variances	
F, DFn, Dfd	1.379, 17, 17
P value	0.5149
P value summary	ns
Significantly different ($P < 0.05$)?	No

Mate preference assay statistical analysis	
Unpaired *t*-test table of CON vs PD males in response to 10mM ascr#3	
P value	0.2993
P value summary	ns
Significantly different ($P < 0.05$)?	No
One- or two-tailed *P* value?	Two-tailed
t, df	t=1.049, df=50
How big is the difference?	
Mean of CON (A)	0.1016
Mean of PD (B)	0.1956
Difference between means (B - A) ± SEM	0.09407 ± 0.08970
95% confidence interval	-0.08609 to 0.2742
R squared (eta squared)	0.02152
F test to compare variances	
F, DFn, Dfd	2.901, 25, 25
P value	0.0099
P value summary	**
Significantly different ($P < 0.05$)?	Yes

Mate preference assay statistical analysis	
Unpaired *t*-test table of CON vs PD males in response to 100mM ascr#3	
P value	0.2789
P value summary	ns
Significantly different ($P < 0.05$)?	No
One- or two-tailed *P* value?	Two-tailed
t, df	t=1.116, df=18
How big is the difference?	
Mean of CON (A)	0.2119
Mean of PD (B)	0.3469
Difference between means (B - A) ± SEM	0.1350 ± 0.1209
95% confidence interval	-0.1191 to 0.3891
R squared (eta squared)	0.06476
F test to compare variances	
F, DFn, Dfd	1.835, 9, 9
P value	0.3793
P value summary	ns
Significantly different ($P < 0.05$)?	No

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Department of Biology, Syracuse University, Syracuse, NY 13244 2015 to 2017
Undergraduate Researcher, Hall Lab

- I conducted behavioral assays to understand the effect of early environmental stresses such as starvation on mating behavior later in development.
- I presented my findings at the Undergraduate Research Symposium at Syracuse University.

Department of Biology, Syracuse University, Syracuse, NY 13244 2015 to 2017
Lab Assistant, Hall and Maine Labs

- I prepared nematode growth media, buffers and solutions, archived worm and bacteria strains, and other miscellaneous tasks.

TEACHING EXPERIENCE

Syracuse University, Syracuse, NY August to December 2017-2019
Graduate Teaching Assistant, Department of Biology

- Teaching assistant for Genetics Lab, an undergraduate course averaging 22 students per semester.
- Set up for various classes and experiments including gene mapping, phenotypic analysis, transformation, complementation, and an introduction to molecular biology, maintained required nematode and yeast strains necessary for the lab.
- Provided support to the instructor during lab sessions and outside lab during office hours to help and mentor students.

Syracuse University, Syracuse, NY January to May 2018-2019
Graduate Teaching Assistant, Department of Biology

- Teaching assistant for Integrative Biology Lab, an undergraduate course averaging 180 students per semester, and about 22 students per lab section.
- Lead lab sections and taught course material related to quantitative skills and data analysis.
- Set up material for cellular and developmental based labs and provide mentorship to students during lab period and office hours.

Say Yes to Education, Syracuse, NY September 2013 to May 2017
SAT Math Instructor, SAT Prep Program

- Tutored high school students from different cultural backgrounds and mentor them for the SATs
- Prepared lesson plans that are flexible depending on the sessions' schedules.
- Monitored proctored exams and provide mentorship to students.

Northside CYO, Syracuse, NY February 2013 to May 2013
Youth Mentor

- Tutored high school and middle school students from different cultural backgrounds and provide help with homework.
- Started free regents prep classes for high school students, geared mainly towards ESL students.

SCIENTIFIC PRESENTATIONS

Oral Presentations:

R. A. Al-Saadi, J. Luo, D. S. Portman, S. E. Hall. *C. elegans* alters mating strategies following early environmental stress. Upstate New York *C. elegans* Meeting, SUNY Upstate Medical University, Syracuse, NY, April 27th, 2019.

Poster Presentations:

R.A. Al-Saadi, J. Luo, D. S. Portman, S. E. Hall. *Caenorhabditis elegans* alters mating strategies based on developmental history. 22nd International *C. elegans* Conference, University of California, Los Angeles, CA, June 19th-23rd, 2019.

R.A. Al-Saadi, N. Wagner, S.E. Hall. N2 Bristol Alters Mating Strategies in Postdauer Adults. Upstate New York *C. elegans* Meeting, Syracuse University, Syracuse, NY,

March 24th, 2018 and Neuroscience Research Day, Syracuse University, Syracuse, NY, April 6th, 2018.

R.A. Al-Saadi, M.C. Ow, S.E. Hall. Developmental History Affects Outcrossing Rates via RNAi in *Caenorhabditis elegans*. Upstate New York *C. elegans* Meeting, SUNY Upstate Medical University, Syracuse, NY, April 29th, 2017.

R.A. Al-Saadi, M.C. Ow, S.E. Hall. The Influence of Developmental History on Outcrossing in *Caenorhabditis elegans*. Neuroscience Research Day, Syracuse University, Syracuse, NY, April 7th, 2017 and Biology Graduate Student Organization Poster Session, Syracuse University, Syracuse, NY, April 26th, 2017.

R.A. Al-Saadi, M.C. Ow, S.E. Hall. Early Environmental Stress Increases Outcrossing in *C. elegans*. Undergraduate Research Symposium, Syracuse University, Syracuse, NY, August 11th, 2016.

HONORS AND AWARDS

The Robert and Susan Hallenbeck Graduate Student Travel Award May 2019
Departmental support for travel expenses related to a research conference at which the student is presenting**.**

22nd International *C. elegans* Conference Travel Award April 2019
A travel award used to offset expenses associated with attending the 22nd International *C. elegans* Conference being held June 20-24, 2019 at the University of California, Los Angeles.

Graduate Student Organization Travel Award April 2019
Departmental support for travel expenses related to a research conference at which the student is presenting.

Research Achievement Award April 2017
This award is given to graduating Biology, Biochemistry, and Biotechnology majors based on their progress in research.

Best Undergraduate Poster Award, Biology Graduate Student Organization Poster Session April 2017

Summer Support Research Award May 2016
Funding is awarded toa limited number of undergraduate researchers to support them while conducting fulltime research in a faculty lab in the Biology Department at Syracuse University.

SERVICE AND OUTREACH

- Judged the Central New York Science and Engineering Fair, March 31st, 2019.

- Judged the SUNY College of Environmental Science and Forestry Environmental Challenge, May 22nd, 2018.
- Participated in a high school outreach event organized by Dr. Kate Lewis, by facilitating experiments done by high school students at Syracuse University, March 7th, 2018.

PROFESSIONAL TRAINING

GENETICS Peer Review Training Workshop
22nd International *C. elegans* Conference, University of California, Los Angeles, CA, 6/2019
This workshop provided an introduction to peer-reviewing for early career researchers and helped to hone skills such as critical thinking, evaluating research, and understanding the process of peer review.

Teaching Workshop: Providing a Broader Research Experience by Collaborating Across Independent CURE Courses
22nd International *C. elegans* Conference, University of California, Los Angeles, CA, 6/2019
This workshop highlighted the importance and potential of Course-based Undergraduate Research Experiences (CURE) in establishing meaningful collaborations across departments and institutions to expand the research conducted by students in a single course.

LAB SKILLS

Worm microinjection
PCR/RT-qPCR
Behavioral assays
DAPI staining
Gene Editing methods such as mosSCI
Molecular cloning
DNA/RNA isolation and purification
Restriction enzyme digests
Agarose gel electrophoresis
Bacterial/yeast transformation
Bacterial culture/streak-plating
Light and fluorescent microscopy
Use of ApE, and GraphPad Prism
Statistical analysis