FITNESS EFFECTS OF A SPONTANEOUSLY ORIGINATING SELFISH MITOCHONDRIAL DELETION FOUND IN *C. ELEGANS*

A Thesis

By

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2022

Major Subject: Biomedical Sciences

ABSTRACT

The mitochondrion goes beyond just the “powerhouse of the cell”. As incredibly important as this organelle is, there is still much about its biology that remains unclear. Mitochondrial variation, historically, has often been treated as a neutral player in the game of evolution, but a growing amount of evidence has made it apparent that mitochondria have played a very active role in eukaryotic evolution. Questions on the mitochondria’s role in topics, such as speciation, adaptation, and disease, are more frequently being investigated. The high mutability of most animal mitochondrial DNA (mtDNA) allows for heteroplasmies to arise and cause mito-nuclear conflict. This genomic conflict has been known to lead to various problems. One such problem is the prevalence of selfish mitochondria that lead to disease. Mitochondrial disease is quite common in humans, so there is particular interest in studying the dynamics of selfish mitochondria. Another issue that can arise is sexual antagonism due to mitochondrial mutations. Due to the maternal inheritance pattern (generally) of mitochondria, it is theorized that males will experience worse fitness costs compared to females. This pattern does exist in various plants and animals, however much of how this phenomenon works is unknown. Typically, animal studies dedicated to the study of sexual antagonism in the context of mitochondrial mutations are conducted in dioecious species, leaving a lack of understanding how this concept might work in hermaphroditic systems. Here we present another selfish mitotype found in *C. elegans* and explore potential sex-specific consequences of the mutant mitochondria.

DEDICATION

“Not all those who wander are lost”
-JRR Tolkien

To my grandparents: Peter and Virginia Sequeira and Jeff and Germaine Barker

ACKNOWLEDGEMENTS

Graduate school has taught me many things, and one of the biggest lessons I learned was that not everything is going to go as planned. Experiments are going to fail, the world will enter a pandemic in your first year, you will experience the worst snowstorm Texas has seen in 100 years, you will decide to earn a Master of Science instead of your PhD. Switching degrees was a difficult decision for me, but ultimately with the support of the people around me, I made the jump.

First and foremost, I would like to thank my wonderful committee, Drs. Vaishali Katju, Ulfar Bergthorsson, Luis Rene Garcia, and Mariana Mateos, who sat through those long meetings and listened to me ramble on about mitochondrial mutations. I am incredibly grateful for you accepting my request to serve on my committee and for your guidance. To my PIs, Drs. Vaishali Katju and Ulfar Bergthorsson, thank you so much for taking a chance on me. When I first reached out to you, I was not very hopeful in entering graduate school, but you invited me for an interview. The opportunity to speak with you both was an enormous boost for me and gave me the confidence that I could still do graduate school. While things did not work out as planned, I am still grateful for the time that I worked as your student. Vaishali, you kept me focused and grounded. Ulfar, you reassured me whenever I was questioning myself. You both taught me so much and I will always look back on our time working together fondly. Lastly, I would like to thank both of you for supporting my decision to switch degrees and pursue new paths. That made going through the application process that much more bearable.

To my lab mates, old and new: (Dr.) Joe Dubie, (Dr.) Thad Deiss, Nick Edenhoffer, Rob Melde, Breann Richey, Austin Daigle, Ian O'Keefe, Amanda Pastrano, Nushrat Rashid, and Aiden Nychka. Joe, thank you for your willingness to help me or talk to me about science. I wish

all the best in your new job! Thad, you are one of the kindest and smartest people I know. I will be forever grateful for your help and advice on all things molecular. I will miss our long conversations about anything and everything. You are one of the easiest people to converse with and I am happy that we were both comfortable to talk to each other about anything. Thank you for all the times that you sat and listened to me rant about whatever it was, I am sure that it was not fun on your part, but I appreciate you lending me your ear. Nick, you are no longer in the lab, but you are doing great things in graduate school. Thank you for all those long philosophical talks that we would have in lab, it was so refreshing, and I miss them. Also, thank you for all the times you stayed late to help me with the last little bit of pipetting, you never had to, but you did anyways. I will not forget that. Rob, you also are no longer in lab, but I am sure you are doing great things up in graduate school as well. I want to thank you for making me feel included and for the times you went out of your way to show me you cared. Bre, even though we only shared a lab for about a year, you made my first year of graduate school so much more manageable. I am so grateful for all the times we would commiserate over problems we might have been dealing with. Austin, thank you for always being willing to listen to my long monologues and for all those times I over shared. Thank you for pushing me to apply for another graduate program last semester, the future is certainly looking brighter now that I took that plunge. To Ian, Amanda, Nushrat, and Aiden, thank you guys so much for all the help you have given me over the past year. You guys made my lab work so much less stressful knowing that I had people who could and were willing to help. Again, thank you to all my lab mates, past and present, without you I would not be the same person I am today. You helped me grow so much!

To everyone that has helped me get where I am today. My friends from undergrad, Kat and Nick Moore, Morgan Flottmeier, Elizabeth Blakely, and so many more, you guys never once

doubted me for a second and have always been my cheerleader when I needed one. We made some incredible memories in college, and I will always cherish those. Reminiscing about UD gave me a bit of comfort during graduate school because I knew I had people who will always stand by me no matter what. To the friends I have made along the way, Veronica Egging, Robert Chemelewski. You guys were some of the first people that I met down here, and I am so glad that I did. I am forever grateful for all the times you just let me have a freak out, but you would always be there to help calm me down. You guys are some of my best friends and I do not think I would be the same person without having known you two. To Dr. Deanna Soper, you are the reason why I even got into research. You gave me so many opportunities to explore my interests in science and because of that I am even more convicted to pursuing the study of human evolution. Thank you so much for taking me under your wing and helping me through my science career, so far.

Lastly, to my family. My parents: Daryl and Joan Sequeira and my siblings: Hannah, Esther, Michal, Sam, Hosea, Reuben, and Martha. Being the first to pursue science is daunting, but I was able to persevere because of you guys. Thank you for your undying support. Thank you for always having my back. I know I rarely visit, but I appreciate that you do not make me feel guilty for not coming by more often. Mom and Dad, thank you for teaching me how to be strong and independent. Thank you for instilling in me an incredible faith and giving me the gift of Catholicism. To all my siblings, I know we fight a lot, but I am never worried about it because I know we are all there for each other. To my grandparents, to whom I dedicate this work, thank you for always showing interest in what I do even if you have no idea what I am talking about.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This works was supervised by a thesis committee consisting of Professors Dr. Vaishali Katju [advisor], Dr. Ulfar Bergthorsson [co-advisor] of the Department of Veterinary Integrative Biosciences, Dr. Rene Garcia of the Department of Biology, and Dr. Mariana Mateos of the Department of Ecology and Conservation Biology.

The original MA lines were generated by Dr. Vaishali Katju. The backcrossed lines utilized were created by Dr. Joseph Dubie. Ian O'Keefe aided in data collection for the competition assay in Chapter II. The rest of the work was conducted independently by the student.

Funding Sources

This work was funded by National Science Foundation grant MCB-1817762 to Drs. Vaishali Katju and Ulfar Bergthorsson.

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CHAPTER I

INTRODUCTION

Mitochondria are essential organelles for eukaryotic life and play an integral role in various cellular functions and metabolism. Most animal mitochondria have a high mutation rate which gives rise to mitochondrial variants and chances for mito-nuclear conflict (Levin *et al*., 2014). Furthermore, mutations can lead to mitochondrial dysfunction which often lead to disease (Duchen, 2004; McBride *et al.*, 2006). Many mitochondrial diseases are associated with mutations in the mitochondrial genome (mtDNA) either arising *de novo* or inherited, generally from the mother (Alston *et al*., 2017) and can vary widely in how the disorder manifests (DiMauro *et al.*, 2013). Moreover, studies have shown that mtDNA diseases are quite common, affecting in one in 5000 humans (Chinnery *et al*., 2000; Gorman *et al*., 2015) and appearing in one in 200 human births (Elliot *et al*., 2008). The majority of mitochondrial diseases affect multiple systems, typically ones that require high energy, such as muscles or the nervous system (DiMauro and Schon, 2001). By studying mitochondrial diseases, we gain deeper insight into the aspects of mitochondrial biology and mito-nuclear evolution that have yet to be elucidated.

The transmission and manifestation of mitochondrial pathologies greatly depends on varying factors, such as genetics, environment, and species. Mutant homoplasmic mtDNA (all mtDNA contain the same mutation within an individual) is inherited by all the maternal offspring, but disease may not always occur. For example, a human mitochondrial disease called Leber hereditary optic neuropathy (LHON) is mainly caused by three mtDNA point mutations and results in blindness for those affected (Mackey *et al*., 1996). LHON is fairly common. ~1 in 8500 individuals have one of the primary point mutations, but about 50% of men and about 10% of women will be affected (Man *et al*., 2003). Moreover, studies have shown an association

between two of the primary mutations and the mitochondrial haplogroup J (Torroni *et al*., 1997; Brown *et al*., 2002). Another well-known example is found in sensorineural hearing loss (SNHL). Most of SNHL cases are caused by a homoplasmic point mutation in the mitochondrial 12S rRNA gene and the level of hearing loss in patients ranges from mild to severe (Prezant *et al*., 1993; Ballana *et al*., 2008). Like LHON, presence of the point mutation does not guarantee disease onset and studies have shown that there is an association with loss of hearing due to the combination of the mutation and administration of amino-glycoside antibiotics (Taylor and Turnbull, 2005). These two examples demonstrate that clinical expression is not solely dependent on harboring a disease associated mtDNA mutation, and that genetic background and environment can play a large role in the onset of mitochondrial disease.

Mitochondrial heteroplasmies (more than one mitotype within an individual) have a complex transmission pattern and phenotypic expression. Heteroplasmies arise in a variety of ways and are found across eukaryotic taxa (Parakatselaki and Ladoukakis, 2021). As part of mitochondrial biology, mitochondria are almost universally inherited maternally and undergo a genetic bottleneck during oogenesis and embryogenesis (Jenuth *et al*. 1996; Cree *et al*., 2008; Wai *et al*., 2008), enabling for variable levels of mtDNA mutations to be transmitted to offspring (Brown *et al*., 2001; Sullins *et al*., 2019). Different species experience different transmission patterns of mitochondrial mutations. For example, in humans, mtDNA deletions are rarely inherited (Chinnery *et al*., 2004). However, in *Caenorhabditis* species, only one duplication has been documented to have occurred (Howe and Denver, 2008) while mtDNA deletions have been found to be inherited by offspring and rise to high frequencies (Clark *et al*., 2012; Konrad *et al*., 2017; Katju *et al*., 2022). Heteroplasmies can also start off at low levels but increase in frequency due to replicative advantage, genetic drift, or new mutations. This phenomenon has

been linked to aging and onset of disease after the deleterious mtDNA reaches a certain threshold, also referred to as a threshold effect (Rossignol *et al*., 2003; Li *et al*., 2015). The threshold effect is an important aspect in the expression of a mitochondrial disease. If the heteroplasmy is below a certain frequency within an individual, then the individual will not display symptoms, but if a threshold frequency (at least 60%) is reached, then the disease phenotype will be expressed (Rossignol *et al*., 2003).

Detrimental mitotypes with a replicative advantage over wildtype mtDNA can be considered selfish mitochondria, in that they can increase in frequency while reducing individual fitness. To designate a mitochondrial variant as selfish, two criteria must be met: 1) it must have negative or neutral fitness effects, and 2) a replicative/transmission advantage (Hurst and Werren, 2001; Phillips *et al.*, 2015). Selfish mitochondria have been recorded in several species including yeast (MacAlpine *et al.*, 2001; Taylor *et al.*, 2002), nematodes (Howe *et al.*, 2010; Dubie *et al.*, 2020), plants (Barr *et al.*, 2005), *Drosophila* (Ma and O'Farrell, 2016), and mammals (Frank and Hurst, 1996). Studies in yeast and nematodes are most notable for easily manipulating population sizes to determine the transmission patterns of putative selfish mitochondria. These studies have demonstrated that under relaxed individual selection, selfish mitochondria increase in frequency more readily each generation, and in some cases, can be maintained in large populations without competition from other fitter mtDNA (Dubie *et al.*, 2020). mtDNA undergo selection at the intra- and intercellular levels regardless of population size, and very few studies have explored the mutation-drift-selection balance in terms of selfish mitochondria proliferation.

It has been proposed that the uniparental inheritance of mitochondria serves as a mechanism for suppressing the transmission of selfish mitochondria is the evolution of uniparental inheritance (Radzvilavicius, 2021). Uniparental inheritance reduces intracellular selection by

only allowing one parent to transmit their mtDNA, increasing the possibility of homoplasmic mtDNA in offspring and decreasing mito-nuclear disruptions caused by heteroplasmies. This also implies there is unisexual selection against whichever sex is not passing on their mitochondria. The maternal inheritance pattern for mitochondria opens the possibility for sexual antagonism against males (Trivers and Burt, 2009). Indeed, this has been observed in *Drosophila* (Patel *et al*., 2016), humans (Wallace *et al*., 1988), mice (Nakada *et al*., 2006), and flowering plants (Schnable and Wise, 1998). Changes in male fitness due to mtDNA is best understood through cytoplasmic male fertility (CMS) in plants (Schnable and Wise, 1998). CMS is caused by a mtDNA variant that reduces pollen production and increases seed production. Because the mitochondria are inherited through the seed, the pollen production diminishes or stops. In wild populations, compensatory mutations in the nuclear genome have been found to rescue pollen production revealing insights into genomic conflict and how mito-nuclear conflicts can be resolved (Budar *et al*., 2003). In animal cases, how mitochondrial variants affect male fitness, but not female fitness is largely unknown (Havird *et al*., 2019).

C. elegans is a useful model organism that allows us to test for selfish mtDNA and their evolutionary implications. *C. elegans* are well known for being hermaphrodites that can self-fertilize and facultatively outcross with males. The ability for self-fertilization enables us to eliminate individual selection by maintaining small population sizes via bottlenecking each generation over time in what is known as a mutation accumulation (MA) experiment. In this thesis I explore a spontaneous 1034 bp deletion that arose in the *cox-3/nd-4* gene region and a nonsynonymous point mutation in *nd4L* (henceforth Δ*nd-4*) of the *C. elegans* mitochondrial genome to a frequency of 81.7% and 93.7%, respectively, in less than 35 generations during an MA experiment (Katju *et al*., 2022). I will discuss the fitness impacts of Δ*nd-4* at both the

individual and large population levels, as well as the proliferation and maintenance of the deletion. Finally, I will also examine any possible sex-specific effects that the Δ*nd-4* mutation might express.

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CHAPTER II

ANOTHER ONE FOR THE BOOKS: THE FITNESS CONSEQUENCES OF A SELFISH MITOCHONDRIA IN *CAENORHABDITIS ELEGANS*

2.1 Introduction

Mitochondria are essential to life, providing a secondary genome for eukaryotes that plays a key role in the evolution of life. The mitochondrial genome (mtDNA) varies across species, ranging in size and the number of polypeptides it encodes for (Lang *et al*., 1999). In most animal mtDNA, there are 13 protein-coding genes which only encode for a fraction of mitochondrial function. While it is most easily recognized for ATP production, the mitochondria has played a critical role in the evolution of eukaryotes by impacting fitness (James and Ballard 2003; Chang *et al*., 2016; Dubie *et al*., 2020), speciation (Webb *et al*., 2011; Burton *et al*., 2013; Telschow *et al*., 2019), evolution of sex (Havird *et al*., 2015; Guerra *et al*., 2017; Nagarajan-Radha *et al*., 2020), and aging (Pinti *et al*., 2014; Dolcini *et al*., 2020).

The mitochondrial genome has interesting population dynamics. Multiple mtDNA exist at the organellar, cellular, individual, and population level in what is called a "nested hierarchy", creating an important mutation-drift-selection balance (Rand 2001). The spontaneous mutation rate of most mtDNA is high (ranging between 0.76 and 1.6 x 10^{-7} per site per generation), which produces heteroplasmies within individuals and are passed to the next generation even at low frequencies (Payne *et al*., 2012; Konrad *et al*., 2017). Heteroplasmies cause variation on which selection can act. Moreover, due to the mitochondria's general matrilineal inheritance pattern, drift occurs each generation, creating multiple rounds of bottlenecking: during oogenesis (Jenuth *et al*., 1996; Wai *et al*., 2008) and embryogenesis (Cree *et al*., 2008). Interestingly, the

bottlenecking of mitochondria works to improve host fitness and increase population variation, while reducing the effects of Muller's Ratchet at large population sizes (Bergstrom and Pritchard 1998). Essentially, as mutations occur, mtDNA variation will increase, allowing for selection to act from the cellular to the individual level as the mtDNA segregates into oocytes and is eventually passed onto the next generation. Studies on the relationship between selection and mutation reveal that directional evolution of the mtDNA is both prevented and occurring. As heteroplasmies occur, purifying selection will remove the mutant genomes before they can be inherited (Wei *et al*., 2019), and positive selection can drive directional evolution through adaptation (Mishmar *et al*., 2003; Cheviron and Brumfield 2009; Lajbner *et al*., 2018).

Heteroplasmies may also classify as "selfish" variants in which certain mitotypes increase in frequency and have a replicative advantage when compared to the wild type (WT) molecules despite them being neutral or deleterious to the individual (Hurst and Werren 2001; Phillips *et al*., 2015). One of the first studies of selfish mtDNA was done in *Saccharomyces cerevisiae*. The *petite* mutation in yeast is a disadvantageous mitochondrial mutation that rose to high frequency in yeast cells at low population sizes, and selection was observed to act both within and among cells (MacAlpine *et al*., 2001; Taylor *et al*., 2002). In a study on *Caenorhabditis briggsae*, novel mitochondrial mutations were more frequent in smaller population sizes, but the specific strain affected transmission patterns of certain mitotypes, suggesting that certain nuclear backgrounds are more susceptible to mutant mitotypes (Phillips *et al*., 2015). Deletions in the *Caenorhabditis* mitochondrial genome are interesting to study because they have been found to persist for multiple generations and display selfish characteristics (Howe and Denver 2008; Estes *et al*., 2011; Dubie *et al*., 2020). More

importantly, they allow scientists to elucidate the possible replicative mechanisms of selfish mtDNA that are still not well understood (Havird *et al*., 2019).

As we attempt to understand mitochondrial heteroplasmies and the impacts they have on the individual level as well as the inter- and intra-cellular level, it is important to study how selfish genetic elements can escape negative selection and persist in populations. *C. elegans* provides a model system that allows for this type of study because of the ability to explore mito-nuclear interactions through spontaneous deletions that accumulate during a bottlenecking regime (Konrad *et al*., 2017). In the current study, a large 1034 bp frameshift deletion spontaneously occurred in an MA *C. elegans fog-2* line (**Figure II-1A, B**; Katju *et al*., 2022) that spans the 3' end of *cox-3*, a tRNA-*thr*, and the 5' end of *nd-4*. This heteroplasmic deletion rose to 81.7% frequency as did a nonsynonymous point mutation in *nd4L* (henceforth Δ*nd-4*). Here we analyze the mitotype's impacts on fitness, population dynamics, and test for selfishness.

2.2 Material and Methods

2.2.1. Isolation of Δnd-4 mitochondria into an N2 background

To directly test phenotypic effects of the mutant mitochondria, the Δ*nd-4* mitotype was backcrossed into a WT *N2* nuclear background. *N2 C. elegans* are self-fertilizers, but can facultatively outcross, and act as the WT reference strain in *C. elegans* studies (Nigon and Felix, 2017). Four *fog-2* lines bearing the Δ*nd-4* mitotype were generated and progeny from three of these lines (A, B and C) were backcrossed for four generations with three WT *N2* males to move from outcrossing to selfing. *Fog-2* strains result from a loss-of-function mutation [*fog-2(lf)*] in the *fog-2* gene, disrupting the genetic pathway for spermatogenesis in hermaphrodites. Mating *fog-2* females with *N2* males over a couple of generations will remove the *fog-2(lf)* and restore

the ability for individuals to self-fertilize. Utilizing hermaphroditic lines allows for simplicity when performing experiments. Frozen lines were thawed, and for ten generations, a hermaphrodite was crossed with three *fog-2* males. Each generation, the parent worms were screened via PCR (forward primer: 5'-AGTACCAGTACACGAGTTGGG-3', reverse primer: 5'-AGAAGGTGGTACACCCCTATTTG-3') to confirm the Δ*nd-4* mitotype was still present. The PCR products were run on a 1% agarose gel (250 ml 1x tris-acetate EDTA; 1g agarose; 1µl GelRed) at 105V for 45 minutes. The expected band sizes were 490 bp for the mutant band and ~1500 bp for the WT.

2.2.2. Fitness Assays

Four fitness assays were performed on three backcrossed lines with the Δ*nd-4* mutation. The four life-history traits tested were developmental rate, survivorship to adulthood (survivorship), productivity, and longevity. Before the fitness assays were conducted, frozen stocks of WT *N2* (control) and the experimental lines (A, B and C) were thawed, and a parental generation was established for all lines. Four days later, the F1 generation was established by setting up 20 replicates for four control *N2* lines (n = 80) and 15 replicates for each of the three experimental lines (A-C) (n = 45). This was done by placing a single L4 hermaphrodite on a 35mm NGM agar plate seeded with *E. coli* OP50. To rid the worms of potential freezer effects, an L4 hermaphrodite was transferred for each replicate onto a new plate every four days for three generations. The F4 generation was used to conduct the assay.

For the survivorship assay, ten L1s were sequestered onto a 35mm NGM agar plate seeded with *E. coli* OP50. This was done for each replicate. 48 hours later, the number of worms that were L4 or older were considered to have survived to adulthood. To assess survivorship, the

number of adult worms was divided by the total number of worms on the plate. Survivorship values can range between 0 and 1.

To measure development rate, a single L1 was placed on a 35mm NGM agar plate seeded with *E. coli* OP50. For each line 15 individuals were used. 36 hours later each worm was assessed every two hours for 24 hours or until the individual reached adulthood. Adulthood was counted at the observation of the first egg entering the uterus. After the first 24 hours, any worms that had not reached adulthood were checked every four hours. Development rate was calculated by taking the inverse of time in hours starting from the L1 larval stage for a worm to reach adulthood.

To measure productivity, the worms from the developmental rate assay were used. Each worm was placed on a 35mm NGM agar plate seeded with *E. coli* OP50 and allowed to lay eggs. The next day the mother worm was transferred to a new plate and then to another the next day. This was repeated each day for eight days. After the mother worm was removed from each plate, the plate was kept in a 20˚C incubator for 24 hours. After 24 hours, the plate was put in a 4˚C refrigerator. The plates were kept at 4°C for three weeks, after which the progeny were counted. Counting was facilitated by adding 200µL of 0.075% Toluidine blue to the plate and, which stains the agar but not the worms.

The same worms used for the development rate and productivity assay were used for longevity. After the last day of productivity, the worm was moved to a new NGM agar plate seeded with *E. coli* OP50 and kept there. Each worm was checked every day until the worm died. An individual was considered dead when the worm was no longer moving after being lightly prodded or there was no pharyngeal activity observed. Longevity was calculated by the number of days the worm was alive, from the L1 larval stage to death.

2.2.3. Fitness Data Analysis

For each of the four fitness assays, data analysis was done using the program R. The relative fitness values for each trait in the mutant lines were calculated according to the mean absolute fitness of the *N2* control lines which were normalized to 1. The fitness measurements were determined by dividing the mean fitness of each trait observed in the mutant lines by the mean fitness observed in the control lines.

2.2.4. Competition Assays

Frozen stocks of three backcrossed lines bearing the Δ*nd-4* mtDNA in a WT nuclear background (A, B and C) and *N2* WT lines were thawed. To remove freezer effects, individual worms were transferred to NGM agar seeded with *E. coli* OP50 plates for two generations. The F3 generation were used to set up 12 populations.

To explore the fitness consequences of the Δ*nd-4* mitotype under competitive conditions we conducted a competition experiment. We established six competed populations (A1, A2, B1, B2, C1, C2) that consisted of equal Δ*nd-4*:WT ratios (50 Δ*nd-4* and 50 WT individuals) onto 100mm NGM plates seeded with an *E. coli* OP50 lawn (1 mL). We also established six uncompeted populations (NCA1, NCA2, NCB1, NCB2, NCC1, NCC2) of 100 Δ*nd-4* bearing individuals onto 100mm NGM plates seeded with an *E. coli* OP50 lawn (1 mL). Every four days, each population underwent the standard *C. elegans* bleaching protocol with a 30% bleach and 15% 5M NaOH solution. The bleaching regimen allowed us to synchronize each generation by only transferring the eggs, thus prevent previous-generation adult worms from contributing to the gene pool. We continued to bleach each population every four days for 15 generations. The

uncompeted populations were maintained for a total of 60 generations following the same bleaching protocol.

To determine the frequency of Δ*nd-4* bearing individuals present in the 12 populations, 30 adult *C. elegans* were randomly selected each generation for a total of 15 generations and subjected to a single worm lysis protocol to extract DNA. The single worm lysis product was used for genotyping via PCR. Three primers were used to determine the presence of the Δ*nd-4* mutation, one forward and two reverse, with the second reverse primer located within the deletion: 5'-AGTACCAGTACACGAGTTGGG-3', 5'-AGAAGGTGGTACACCCCTATTTG-3', and 5'-AATTCTAACAAAGCTACTAGAAACCTT-3' respectively. Individuals bearing the mutation were expected to display both the mutant band (490 bp) and the WT band (350 bp) since Δ*nd-4* bearing worms are heteroplasmic for the mutant mitotype. WT individuals were expected to display only the WT band (350 bp). The PCR products were run on a 1.5% agarose gel (250 ml 1x tris-acetate EDTA; 3.75g agarose; 1µl GelRed) at 105V for 45 minutes.

2.2.5. Calculating relative fitness of Δnd-4 at high population

In addition to calculating the frequency of Δ*nd-4* bearing individuals per generation, the composite relative fitness, *w*, was also calculated. The ratio *p/q*, where *p* is the average frequency of Δ*nd-4* bearing individuals and *q* is the average frequency of WT individuals is equal to *w*. The log of the ratio (*p/q*) per generation (*t*) for each plate was calculated. The log of the ratio was taken for each generation and a linear regression was performed as a function of generational time. The slope of the regression line, *b*, equals log(*w*). By taking 10^b, we can solve for *w*.

2.2.6. Testing for Replicative Advantage

To test for selfish drive, we measured the Δ*nd-4* frequency of 15 (*n* = 45) individuals randomly selected from the uncompeted populations, NCA1, NCA2, NCB2, NCC1, and NCC2. Individuals that had an intermediate Δ*nd-4* frequency (30-40%) would be used in the experiment. Only one individual from population NCA1 was found to have an intermediate frequency of 34%. The progeny were bottlenecked via single worm transfer each generation for ten generations. On generations one, five, and ten, the mothers (*n* = 15) were allowed to lay eggs for a couple of days and then lysed to determine its mutation frequency via digital droplet PCR (ddPCR). For ddPCR, the single worm lysates were diluted 1:50 and prepped in a 96-well plate. Two Bio-Rad ddPCR fluorescent probes (FAM and HEX) were used to determine frequency of WT to Δ*nd-4* mtDNA in each sample. The FAM probe targeted Δ*nd-4* mtDNA, by amplifying a mtDNA segment inside the deletion and the HEX probe targeted WT mtDNA. Probe designs are proprietary but see **Table II-1** for the amplicon context to build the probes. Δ*nd-4* mtDNA frequency was calculated by dividing the concentration of FAM by the concentration of HEX and subtracting that value from one.

2.3 Results

2.3.1. A deletion spanning two genes was detected at high frequency

A 1034 bp frameshift deletion (**Figure II-1A**) starting in the 3' end of the *cox-3* gene and ending in the 5' end of *nd-4* gene was observed to arise spontaneously in a *fog-2 C. elegans* line during an MA experiment (Katju *et al*., 2022). The deletion was estimated to have risen to a frequency of 81.7% and included 11.4% of *cox-3*, 100% of *tRNA-thr*, and 68.2% of *nd-4* (**Figure II-1B**). The complete loss of a tRNA could cause translation disruption during mitochondrial protein production. Along with the deletion, there was also a nonsynonymous point mutation

(Leu → Pro) in the *nd4L* gene that rose to a 93.7% frequency (Katju *et al*., 2022). Together, we refer to this mitotype as Δ*nd-4*.

2.3.2. The Δnd-4 deletion causes significant fitness reduction

To test for fitness effects due to the Δ*nd-4* mutation, four fitness assays were used: development rate, longevity, productivity, and survivorship to adulthood (also referred to as survivorship). These four assays were tested in three mutant lines, A, B, and C (n = 45), and four wild type control lines (n = 80). The relative fitness of the three mutant lines was found to be significantly decreased relative to the wild type (**Figure II-2**).

Development rate is determined by taking the inverse of time in hours that it takes for an individual to grow from the L1 larval stage to adulthood. The Δ*nd-4* bearing nematodes had a significant decrease in development rate compared to the WT *N2* (**Figure II-2**; Wilcoxon Rank Sum, z = -7.07, $d.f.$ = 1, $p < 0.0001$). The average development time (hours) of Δ*nd-4* bearing lines and WT lines was 66.25 hrs and 47.57 hrs, respectively. This means that mutant bearing worms took, on average, 18.7 hours longer to reach adulthood, representing a 39.3% increase in development time. Moreover, the development rate of the Δ*nd-4* bearing worms dropped by 29%. There was no significant difference between the three mutant lines (Kruskal-Wallis Rank Sum, X^2 = 0.7631, $d.f.$ = 2, p = 0.69).

Based on the longevity assay, the life span of mutant-bearing lines was determined to be significantly lower than the WT control lines (10.98 days versus 14.81 days, respectively) (**Figure II-2**; Wilcoxon Rank Sum, z = -3.12, $d.f.$ = 1, p = 0.0018). The mutant worms lived, on average, 3.83 fewer days than the WT control worms. This corresponds to 25.9% decrease in

longevity. There was no significant difference in longevity between the mutant lines (Kruskal-Wallis Rank Sum, $X^2 = 0.6174$, *d.f.* = 2, $p = 0.73$).

For productivity, the mutant lines were significantly different from the WT control lines (**Figure II-2**; Wilcoxon Rank Sum, $z = -8.43$, *d.f.* = 1, $p < 0.0001$). The mutant lines had, on average, a 63.7% decrease in productivity compared to the WT control lines. Δ*nd-4* bearing lines productivity ranged between 96.8 and 133.64 progeny over the course of eight days. There was no significant difference between the three mutant lines (Kruskal-Wallis Rank Sum, $X^2 = 2.498$, *d.f.* = 2, $p = 0.29$).

The mutant lines' survivorship was significantly different from the WT control lines (**Figure II-2**, Wilcoxon Rank Sum, $z = -9.12$, *d.f.* = 1, $p < 0.0001$). The survivorship of the mutant lines, on average, was about 34% lower than the wild type controls. On average, only 49% of L1 larvae survived to adulthood in Line C, whereas survivorship in Lines A and B ranged from 71-75%. Line C was significantly different from lines A and B, but A and B were not significantly different from each other (Kruskal-Wallis Rank Sum, $X^2 = 9.5233$, *d.f.* = 2, $p = 0.01$).

2.3.3. Severe fitness decrease in Δnd-4 bearing worms when maintained at high population sizes

To explore the population dynamics of Δ*nd-4*, a competition assay was performed on the three backcrossed lines with two replicates (six assays), competing Δ*nd*-4 harboring worms with worms containing wild type mtDNA. In addition, two replicates of each backcrossed line were maintained for 60 generations at large population sizes. There was a sharp decrease in frequency of Δ*nd-4* bearing worms in all six competition experiments (**Figure II-3A**). Individuals with the Δ*nd-4* mitotype went extinct in lines B1, B2, C1 and C2, while individuals with the mutation in

lines A1 and A2 persisted at low frequencies until the end of the competition experiments (**Figure II-3A**).

Along with the competition plates, six lines of uncompeted plates were maintained at high population sizes. Unlike the mutation-bearing worms in the competition plates, the Δ*nd-4* was maintained at 100% frequency across all uncompeted lines each generation, suggesting that the extinction or decrease of mutant-bearing worms in the competitive populations was due to a decrease in fitness of the Δ*nd-4* carrying worms and not due to loss of Δ*nd-4* within worms (**Figure II-3B**).

We calculated the relative fitness, *w*, by taking the log of the ratio (*p/q*) over each generation, *t*, and performed a linear regression as a function of generational time (**Figure II-3B**). The $\log(p/q)_t$ had a sharp decrease across each generation and the slope of the regression line, *b*, was -0.23. The slope is equal to log(*p/q*), so *w* was ~0.60. From *w* we were able to calculate the negative selection coefficient to be 0.40. This means that the Δ*nd-4* bearing worms had a 40% composite fitness decrease in a competitive environment. Interestingly, by generation 60, a majority of the uncompeted populations (NCA1, NCA2, NCB1, NCB2, NCC1, NCC2) exhibited a decrease in Δ*nd-4* to extremely low frequencies, with only NCA1 and NCC2 maintaining a median frequency of ~24% and 17%, respectively (**Table II-2**).

2.3.4. Selfish Drive

To test whether genetic drift or selfish drive explains the rise of Δ*nd-4*, we established a parental generation with a 34% Δ*nd-4* frequency. We generated 15 lines from these intermediate frequencies and propagated them via single worm transfer. We calculated the change in frequency over the course of ten generations and found the mutation to have risen to an average

of 77%. The 43% increase from the parental generation is significantly different (Student's: t = -16.4, $p < 0.0001$). These results suggest the mutant mitotype has a replicative advantage.

2.4 Discussion

The coevolution of the mitochondrial and nuclear genomes has led to tight mito-nuclear interactions that are important to eukaryotic life (Lang *et al*., 1999). Mitochondrial mutations introduce heteroplasmies into the mitochondrial population and disrupt these interactions when the variant reaches a high enough frequency leading to varying phenotypic effects (Dowling *et al*., 2008). Selfish mitochondria are important for understanding the evolution of mitochondria and how mitochondrial disease can persist in eukaryotes. The Δ*nd-4* mitotype rose to a high frequency of 81.7% during a mutation accumulation experiment that used an obligately outcrossing *fog-2 C. elegans* line. The main focus of this study was to determine whether or not the Δ*nd-4* mutation is selfishly acting.

The Δ*nd-4* indel created a frameshift in the *cox-3/nd-4* gene region in the mtDNA as well as deleting a tRNA-*thr*. The deletion removed 11.4% of the *cox-3* gene, a tRNA-*thr*, and 68.2% of the *nd-4* gene (**Fig. II-1B**). The Δ*nd-4* mitotype also contained a nonsynonymous point substitution (Leu → Pro) in the *nd4L* gene that rose to a frequency of 93.7% (Katju *et al*., 2022). According to known thresholds for point mutations, pathogenesis is reached at ~90% (Rossignol *et al*., 2003). This means that the point substitution could contribute to the decline in fitness. However, due to the experimental lines being frozen only at the start and end of the MA, there is no way to determine how much the *nd4L* point mutation adds to the fitness effects independent of the deletion since it is not possible to determine if the point substitution arose before or after the deletion. As stated earlier, both mutations are considered as the Δ*nd-4* mitotype, and we found that there was a significant decrease in fecundity, survivorship,

longevity, and developmental rate (**Fig. II-2)**. Individuals harboring Δ*nd-4* are also rapidly outcompeted in mixed populations with WT worms and have a 40% decrease in composite fitness (**Fig. II-3A**, **B**).

The uncompeted populations were able to maintain the mutation for a short period of time. During the experiment, we observed that the uncompeted populations went through periodic crashes that would take a few generations to reach very large population sizes again. Eventually, the uncompeted populations maintained a stable large population size. After 60 generations, we found that the Δ*nd-4* heteroplasmy ranged from very low frequencies to high frequencies in five out of six populations (NCA1, NCA2, NCB2, NCC1, and NCC2). However, population NCB1 maintained a median 90% heteroplasmic frequency (**Table II-2**). One explanation for these results could simply be that at consistently higher population sizes, the power of selection increases and can more efficiently reduce the level of detrimental mitotypes. Alternatively, compensatory mutations in the nuclear genome could have arisen to ameliorate the detrimental effects of the mutant mitochondria. It is important to note that availability of resources, such as nutrients, could account for the drastic change in Δ*nd-4* frequency for most of the uncompeted populations. A recent study by Gitschlag *et al*. (2020) found that initial nutrient abundance may unintentionally promote detrimental mitotypes to replicate in the germline, but overall lack of nutrients promotes WT mtDNA replication over the selfish genotype, meaning that over time the deleterious mtDNA can decrease in frequency (Gitschlag *et al*., 2020). In the case of the Δ*nd-4* uncompeted populations, we could be observing a similar scenario. During the experiment, if the uncompeted population did not experience a population crash, then the NGM plates would be starved by day three in the four-day bleach transfer cycle. It is possible that the nutrient depletion occurred around the time the worms reached sexual maturity. Hence, embryos

that have a lower frequency of the Δ*nd-4* mitotype would be selected for and result in a frequency reduction of the mutant mitotype over time. Because NCB1 conserved a high Δ*nd-4* mitotype frequency, it is possible that the population crashes occurred often enough to maintain a consistent nutrient abundance that kept the Δ*nd-4* mitotype frequency within individuals high. Overall, this may give insight into how nutrition impacts how efficient selection acts upon large populations in which all members share selfish mitochondrial genotypes.

The second criteria for selfish mitochondria are that the genotype must have a replicative advantage over the WT. We took individuals from generation 60 of the uncompeted populations that had a reduced Δ*nd-4* frequency and conducted another MA experiment. We found that within ten generations of bottlenecking via single worm transfer, Δ*nd-4* increased from an initial average 34% heteroplasmic frequency to 77% (**Fig. II-4**), consistent with a replicative advantage over WT. Replication mechanisms of selfish mitochondria are not well understood, but one explanation could simply be faster replication or preferential use of replication machinery (Havird *et al*., 2019). Faster replication or preferential transmission of selfish mitochondria have been implicated in yeast (MacAlpine *et al*., 2001; Taylor *et al*., 2002), nematodes (Clark *et al*., 2012; Phillips *et al*., 2015), humans (Diaz *et al*., 2002; Russell *et al*., 2018), and *Drosophila* (Ma and O'Farrell, 2016). With such a large deletion in the Δ*nd-4* genotype, it is possible that the deleterious mitochondria can replicate faster in the germline and then accumulate over the lifespan of the individual.

In conclusion, we report the identification of another selfish mitotype in the *C. elegans* species. As stated earlier, two conditions need to be fulfilled for a mitotype to be deemed selfish: 1) it must have either negative or neutral fitness impacts, and 2) it must have replicative/transmission advantage. The Δ*nd-4* genotype has been shown to satisfy both criteria.

Hence, we can include this mitotype in the growing list of selfish mitochondria. Furthermore, exploring the relationship between selection and mutations in the context of "selfishness" can lead to better understanding how mitochondrial disease spreads and persists across generations.

2.5 Tables

Table II-1: Description of the ddPCR probes used to calculate the frequency of the Δ*nd-4* mitotype within individuals. Probes were designed by Bio-Rad.

Fluorescent Tag	Amplicon Context	Gene	Amplicon Length
FAM	TTTTTACATCTTTGATTACCTA AAGCTCATGTAGAGGCTCCTA CAACAGCTAGAATACTTTTAG CTGGATTACTATTAAAATTAG GCACAGCGGGATTTTTACGTA TTTTAGGTAGTTTAAGA	*nd-4*	89 bp
HEX	GTCATTTATTGGGAAGAAGAC AAAATCGTCTAGGGCCCACCA AGGTTACATTTATGGGATTAG CACAAGCTTTATTGGATGGGG TTAAACTTTTAAAAAAAGAAC AAATAACACCCTTAAATT	*nd-1*	97 bp

Table II-2: Average fitness of Δ*nd-4* bearing lines in a WT background. 20 replicates for each four *N2* control lines (n=80) and 15 replicates for three Δ*nd-4* bearing lines (n=45) were assayed. Means for each trait was calculated across all control and experimental lines, as well as for each individual line.

	Fitness Traits			
Line	Development Time (hrs)	Longevity (days)	Productivity	Survivorship
Δ*nd-4* bearing lines	66.25	10.98	111.67	0.65
N2 Control	47.57	14.81	307.95	0.99
A	66.83	10.92	104.57	0.71
B	64.92	11.2	96.8	0.76
C	67	10.83	133.64	0.49
N2.1	47.26	16.1	295.79	0.995
N2.2	47.79	14.45	293	0.99
N2.3	47.33	15.35	326.5	0.99
N2.4	47.89	13.35	317.47	0.985

Table II-3: Range and median of Δ*nd-4* frequencies for uncompeted populations, NCA1, NCA2, NCB1, NCB2, NCC1, NCC2. 15 individuals for each population were lysed via single worm lysis and Δ*nd-4* frequency was determined via ddPCR.

LINE	RANGE	MEDIAN
NCA1	0.0-0.905	0.247
NCA2	0.0-0.048	0.0
NCB1	0.812-0.921	0.9
NCB2	0.0-0.0417	0.0
NCC1	0.0-0.0493	0.0
NCC2	0.0-0.922	0.177

2.6 Figures

Figure II-1. Observance and location of Δ*nd-4* in *C. elegans* line. **A**) The read depth of Δ*nd-4* from Illumina whole-genome sequencing mapped to the *cox-3/nd-4* gene region. **B**) A map of *C. elegans* mtDNA (adapted from Okimoto *et al.*, 1992).

Figure II-2. The mean relative fitness for four fitness traits: developmental rate, longevity, productivity, and survivorship. All four fitness assays were conducted across three backcrossed *C. elegans* lines (dark blue) with 15 replicates each (n=45) and four *N2* control lines (red) with 20 replicates each (n=80). The mean relative fitness was calculated for the *N2* control lines and normalized to 1 to compare fitness effects. There was a significant decrease in fitness among all Δ*nd-4* bearing lines compared to the *N2* WT lines. ** $p \leq 0.01$, **** $p \leq 0.0001$.

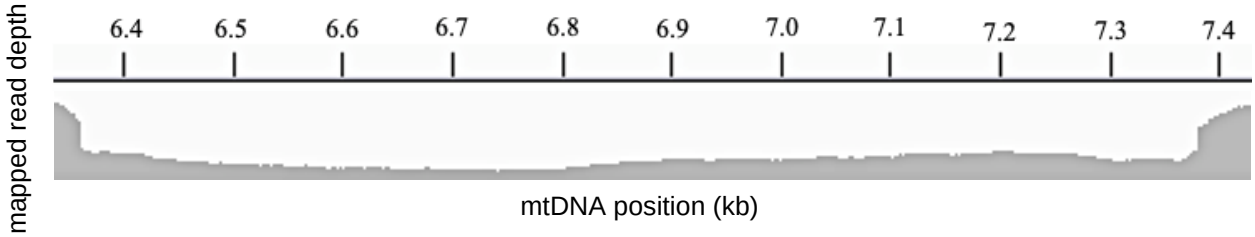
Figure II-3. Competition assays were conducted to determine population fitness effects of the Δ*nd-4* mutation. For each line, two competed replicates were established with an equal Δ*nd-4* :WT ratio and two uncompeted replicates with 100% Δ*nd-4* frequency were generated for each line. **A**) The frequency of the deletion bearing worms had a sharp decline in most competed lines. Over seven generations Δ*nd-4* bearing worms went extinct or near extinct. The uncompeted plates maintained the 100% frequency across each generation. **B**) A linear regression was calculated based on the log (Δ*nd-4*/WT). Each point (orange diamond) represents the average per generation across each competed line (A1, A2, B1, B2, C1 and C2). The relative fitness of the Δ*nd-4* mitotype was calculated from the slope of the regression line and was estimated to be -0.23.

Figure II-4. Offspring from an individual with a 34% Δ*nd-4* heteroplasmy (dotted line) underwent MA via single worm transfer for ten generations. Generation one (red) had an average

frequency of 34%, generation five (blue) 58%, and generation ten (orange) 77%. There was a 43% increase in the Δ*nd-4* deletion from generation one to generation ten.

Figure II-1: Observance and location of Δ*nd-4* in *C. elegans* line.

A)



B)

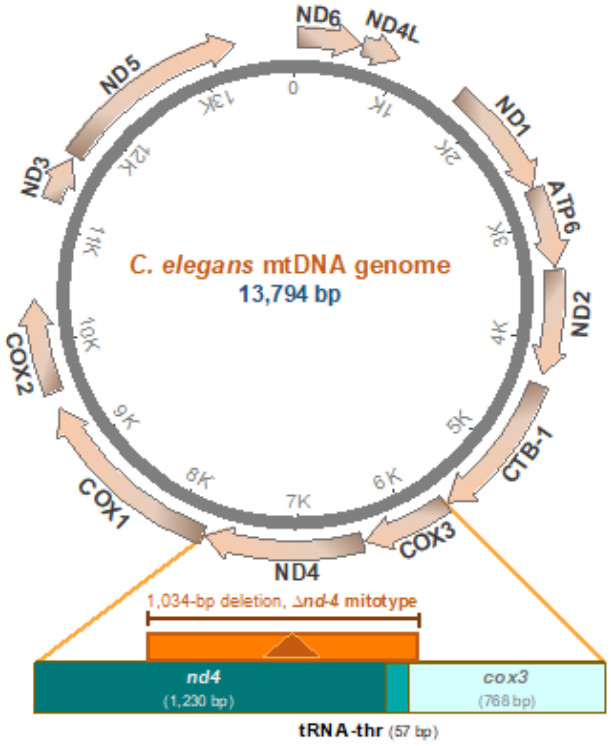


Figure II-2: The mean relative fitness for four fitness traits: developmental rate, longevity, productivity, and survivorship.

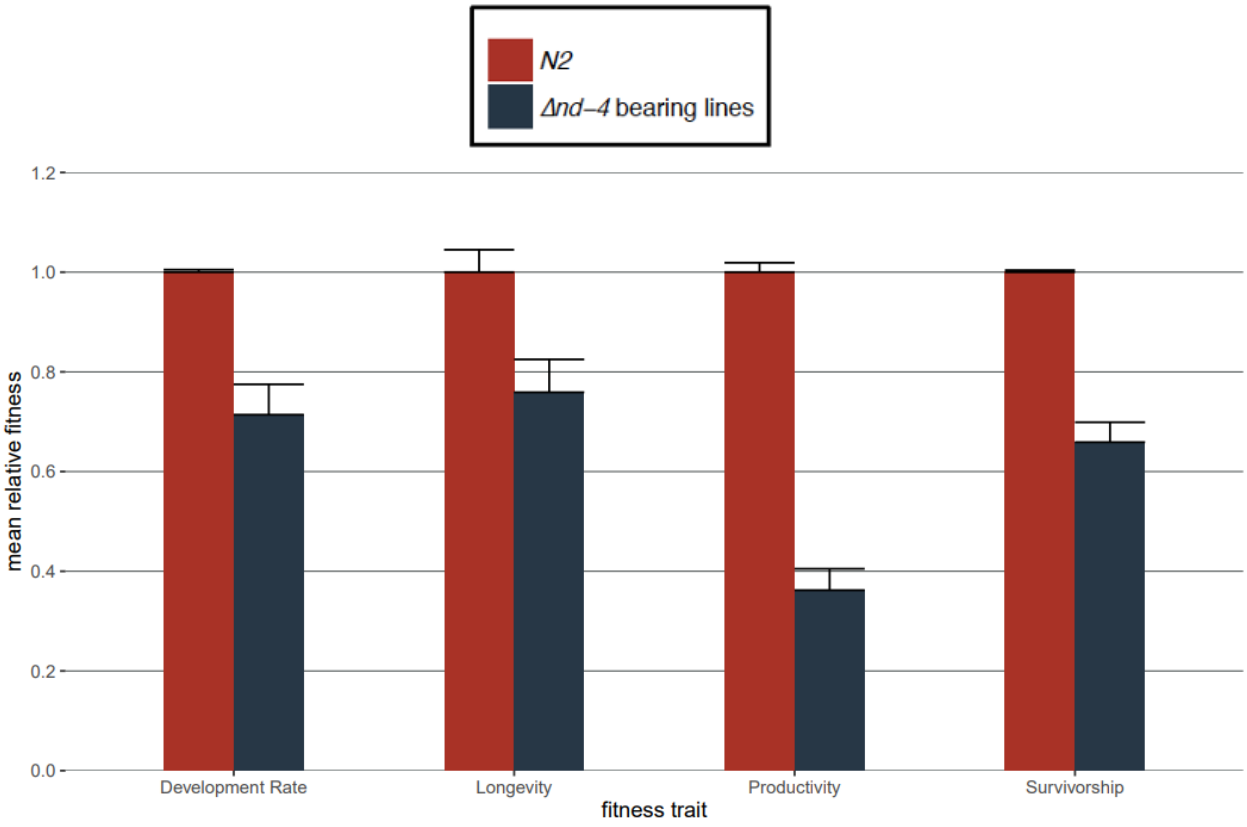
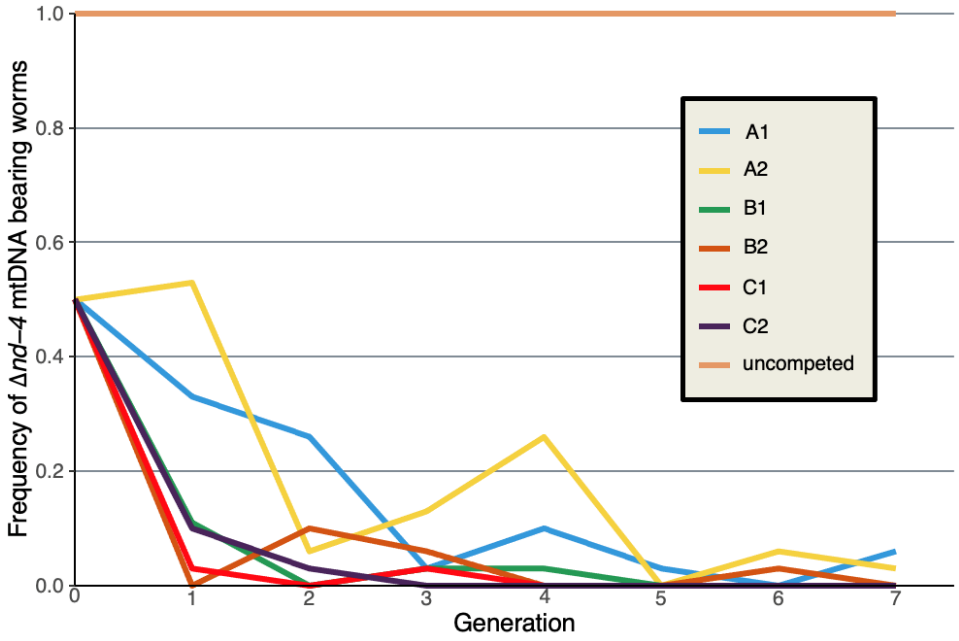


Figure II-3: Competition assays were conducted to determine population fitness effects of the Δ*nd-4* mutation.

A)



B)

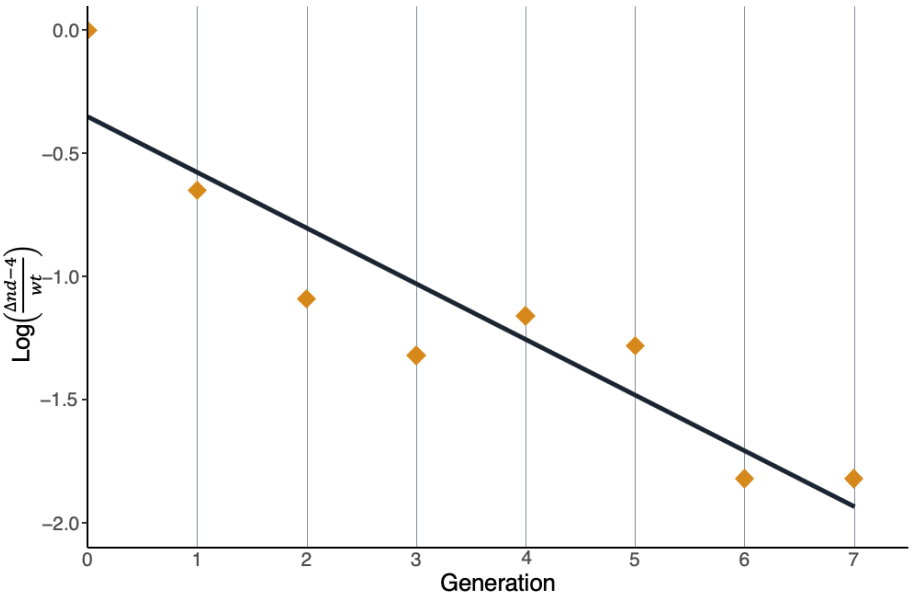
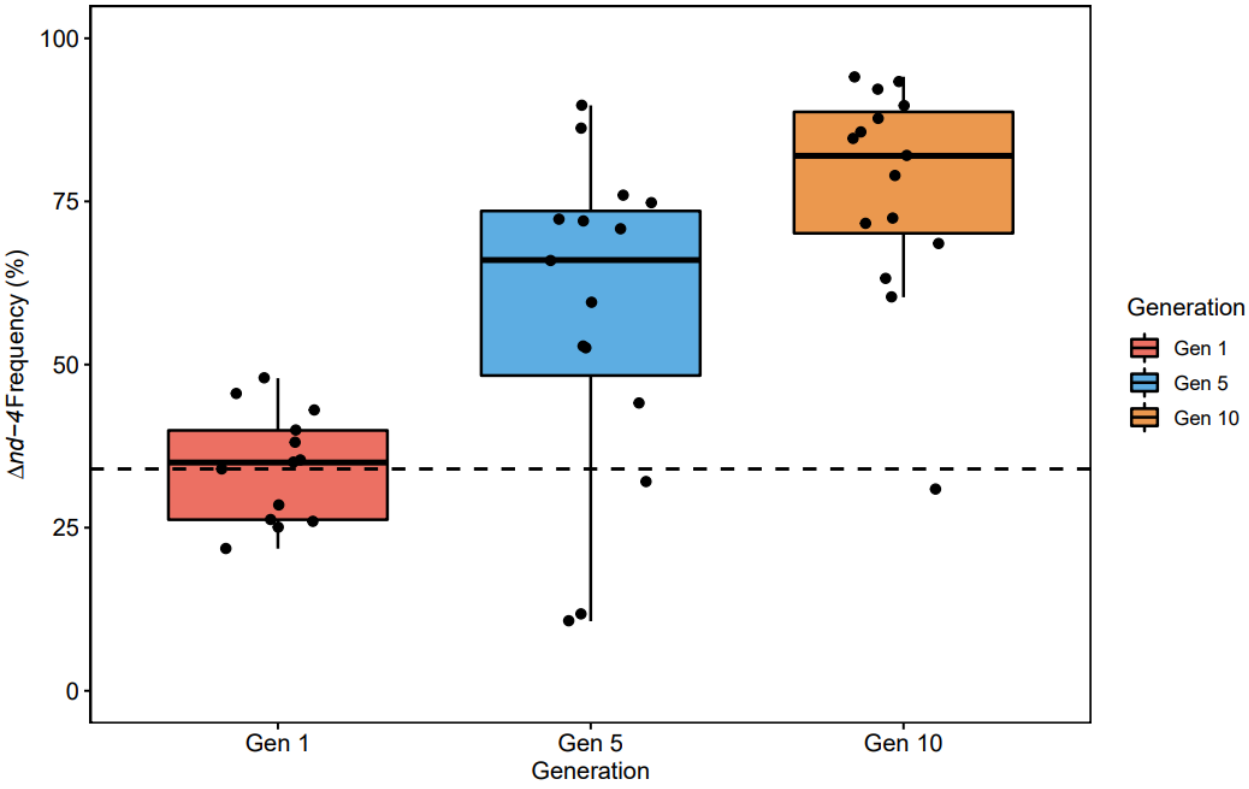


Figure II-4: Offspring from an individual with a 34% Δ*nd-4* heteroplasmy underwent MA via single worm transfer ten generations.



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CHAPTER III

WHAT ABOUT SEX? POSSIBLE SEX-SPECIFIC EFFECTS DUE TO Δ*ND-4* IN *FOG-2 CAENORHABDITIS ELEGANS*

3.1 Introduction

Sexual reproduction is ubiquitous and is a major contributor to the diversification and evolution of eukaryotic life (Payne and Krakauer, 1997; De Aguiar *et al*., 2009). The maintenance of sexual reproduction is a topic that many scientists have explored for decades as it has created various conundrums in the theory of evolution for multicellular eukaryotes. Notably, the twofold cost of males, which states that the birthrate of an asexual individual is twice that of a sexual individual, implying that asexual populations should have a competitive edge over sexual populations (Smith, 1971, 1978). Yet, this is not what we observe. There are many examples of species that have asexual and sexual lineages coexisting in populations (Case and Taper, 1986; Jokela *et* al., 1997; Schon *et al*., 2000; Stelzer, 2011; Combosch and Vollmer, 2013) and sexual reproduction, in its many forms, is found in all major eukaryotic branches (Heitman, 2015). Breeding mode proportions are heavily influenced by population size, mutation-selection balance, and recombination rate in which asexual or sexual reproduction can gain advantage over the other (Takahata, 1982; Kondrashov 1988; Schon *et al*., 2000).

Sexual reproduction has both advantages and disadvantages. The most obvious advantage being that sexual reproduction has the added benefit of recombination, which can separate detrimental allele combinations and increase the population's ability to adapt to changing environments (Barton and Charlesworth *et al*., 1998) whereas asexual reproduction suffers from the effects of Muller's Ratchet via mutation accumulation (Lynch *et al*., 1995). However, the advantage of recombination decreases if a population has reached equilibrium and is at its fittest,

because segregating linked alleles can actually cause a reduction in fitness (Otto and Lenormand, 2002). Other disadvantages in sexual reproduction are found in harmful mating behaviors that can result in death or injury (Andrade, 2003; Morrow *et al*., 2003), and sexual selection that reinforces maladapted traits (West and Packer, 2002; Candolin *et al*., 2008; Martinossi-Allibert *et al*., 2018) and exacerbates sexual antagonism (Holland and Rice, 1998; Connallon and Clark, 2014). Hermaphroditism helps to alleviate some of the disadvantages of outcrossing, such as dangerous mating behaviors, but increases inbreeding depression and can still maintain sexual antagonism (Abbott, 2011; Jordan and Connallon, 2014).

Sexual selection acts differently on males and females, generally acting stronger on males than females (Agrawal, 2001). Sex-specific morphologies in dioecious species are extremely well studied and are foundational in the sexual antagonism coevolution theory (Perry and Rowe, 2015) and sex-specific genetic asymmetries have distinct effects on how strong natural selection is acting on males and females (Connallon *et al*., 2019). However sexual antagonism is less obvious in hermaphroditic systems and relies more on intra-locus sexual conflict. This is because in hermaphrodites, sexual antagonistic alleles are more readily exposed to selection; thus, intra-locus conflict becomes the limiting factor in hermaphrodites as compared to species with distinct sexes (Bedhomme *et al*., 2009; Abbott, 2011).

Another interesting aspect of sexual antagonism is how genomic conflict can affect the evolutionary trajectories of males and females or sex-specific functions. Genomic conflict is an extremely important aspect in evolutionary theory because the study of genetics boils down to how DNA (nuclear and cytoplasmic) interacts, and this conflict is the cornerstone for creating the diversity we see in eukaryotic life (Rice, 2013). Mito-nuclear conflict is of great interest because of strict matrilineal inheritance found in most plants and animals and the sex-specific

effects that can arise subsequently (Cosmides and Tooby, 1981). Simply put, because females are transmitting the mitochondria to the next generation, selection will act on female mitochondrial DNA (mtDNA) potentially leaving males to suffer from mitochondrial mutations that arise to the benefit/detriment of females/males (Frank and Hurst, 1996).

We see this phenomenon manifest in a variety of ways. For example, Innocenti *et al*. (2011) used *Drosophila* to test the hypothesis that male mtDNA should have a higher mutation load and that the degree of fitness effects would be more apparent in species with higher levels of sexual dimorphism because selection will not be acting on male mitochondria (Innocenti *et al*., 2011). They found that the mitochondrial strain greatly influenced the differential expression of genes associated with male tissues and that selection is less efficient at purging mutation build-up that have male-specific effects. Moreover, they found that male sterility was higher when a specific mitochondrial haplotype was paired with an w^{1118} isogenic nuclear background, but males were fertile when that same haplotype coexisted with the original coevolved nuclear background. Selfish mitochondria introduce mito-nuclear conflict that led to well established reproductive manipulation tactics (i.e. cytoplasmic male sterility [CMS]) and less established hypotheses, such as the Mother's Curse (Dowling and Adrian, 2019; Havird *et al*., 2019). CMS is a well-documented case for the Mother's Curse hypothesis in plants and occurs from mtDNA variants reducing the amount of pollen a hermaphrodite can produce and effectively turning it female. Mitochondrial variants skewing the sex ratio has yet to be documented in animals (Havird *et al*., 2019).

In animals, the Mother's Curse is less clear. Vaught and Dowling conducted a meta-analysis on studies involving the Mother's Curse. They found that there is a research bias towards males and results suggesting Mother's Curse dynamics could be more of an artefact of

not exploring female-specific effects rather than evidence for the hypothesis (Vaught and Dowling, 2018). However, they do discuss some putative evidence for the Mother's Curse hypothesis. In *Drosophila*, studies on reproductive traits in males and females found that mtDNA mutations in the cytochrome *b*, and cytochrome oxidase I and II genes result in lowered male fertility but not in females (Xu *et al*., 2008; Clancy *et al*., 2011; Patel *et al*., 2016). Other animal examples can be found in hares (Smith *et al*., 2010), mice (Trifunovic *et al*., 2004; Ma *et al*., 2016), and humans (Martikainen *et al*., 2017).

The study of sexual antagonism due to selfish mitochondrial variants is little understood (Havird *et al*., 2019). Studies have shown sexual antagonism in a variety of *Caenorhabditis* species due to nuclear background or mutations, but under the context of mtDNA driven sexual antagonism, they have been underused (Ancell and Pires-daSilva, 2017). Here we look at possible sex-specific effects of a selfish mitochondrial mitotype that rose to high frequency during a bottlenecking experiment using a line of *fog-2 C. elegans* (Katju *et al*., 2022). The mitotype contained a 1034 bp deletion spanning the 3' end of the *cox-3* gene, a tRNA-*thr*, and the 5' end of the *nd-4* gene and a nonsynonymous point substitution in the *nd4L* gene (henceforth Δ*nd-4*). The current study elucidates possible sex-specific fitness effects due to the Δ*nd-4* mitotype via reproductive assays (productivity and male mating ability) as well as sex ratio and longevity.

3.2 Materials and Methods

3.2.1. Isolation of Δnd-4 in fog-2 C. elegans strain

Four lines bearing the Δ*nd-4* deletion (A-D) were thawed and transferred onto 35 NGM agar plates seeded with *E. coli* OP50. For ten generations, a single *fog-2* female bearing the Δ*nd-4* mutation was crossed with three *fog-2* WT mtDNA males. Because mitochondria are

maternally inherited, this backcrossing regime allows us to isolate the mutant mtDNA into a *fog-2* nuclear background and remove possible line-specific nuclear mutations that coevolved with the Δ*nd-4* mitotype and affect fitness. Each generation, Δ*nd-4* presence was confirmed via PCR. Four separate Δ*nd-4* bearing *C. elegans* lines (A-D) with a *fog-2* nuclear background were generated.

3.2.2 Survivorship to adulthood and Sex Ratio

Four backcrossed *fog-2* lines bearing the Δ*nd-4* deletion (A-D) and a WT mtDNA *fog-2* control were thawed onto individual 35mm NGM agar plates seeded with *E. coli* OP50. For two generations, a single L4 female and two L4 males were transferred to a new plate to remove possible freezer effects. There were 12 replicates for the control F1 and F2 generations. For each mutant-bearing subline (A-D), there were three replicates each for a total of 12 replicates representing the Δ*nd-4* mutation. Ten L1 progeny from the F3 generation were transferred to 35mm NGM agar plate seeded with *E. coli* OP50 for each F2 cross (n = 120). The plates were checked 36 hours later to determine the sex ratio and survivorship to adulthood (survivorship).

3.2.3. Sex-Specific Longevity

We conducted a longevity assay to compare the lifespan of males versus females. The siblings of the individuals used in the survivorship assay were used for the longevity assay. Because sex is visually indeterminate from the L1 to L3 larval stages, F3 individuals were selected for this experiment at the L4 larval stage when sex can be visually distinguished. For each F2 cross, five males and five females were transferred to individual plates (WT mtDNA males n = 60, WT mtDNA females n = 60, Δ*nd-4* males n = 53, Δ*nd-4* females n = 54). The worms were observed each day until death. Death was determined when all movement ceased and there was no reaction to gentle prodding on and around the worm.

3.2.4. Sex-specific Productivity

Backcrossed Δ*nd-4 fog-2* lines (A-D) and WT mtDNA *fog-2* control lines were thawed and populations established on NGM plates seeded with *E. coli* OP50. For two generations, a single L4 female and two L4 males were transferred to a new plate to remove freezer effects. Generation F3 individuals were used to set up four different crosses: *fog-2* female x *fog-2* males (WT mtDNA control), Δ*nd-4* female x Δ*nd-4* males (mutant mtDNA control), Δ*nd-4* female x *fog-2* males, and Δ*nd-4* female x *fog-2* males. Note that for each cross two males and one female were paired. Each cross type had 20 replicates. For each of eight consecutive days, the male/female pairs were transferred to a fresh 35mm NGM agar plate seeded with *E. coli* OP50. The plates were stored in an incubator at 20°C for 24 hours before being placed in a 4°C refrigerator for three weeks. After the three weeks, the number of offspring were counted. Counting consisted of pipetting ~200µl of 0.075% Toluidine blue and then counting the number of worms on the plate under a dissecting microscope. Toluidine blue makes it easier to count the worms as it stains the agar, not the worms.

3.2.5. Male Mating Ability Assay

The male mating ability (MMA) assay enabled us to determine the success rate of male mating and fertilization. Four backcrossed *fog-2* lines (A-D) bearing the Δ*nd-4* mutation and a WT mtDNA *fog-2* control were thawed and placed on 35mm NGM agar plates seeded with *E. coli* OP50. The lines were propagated for two generations to remove freezer effects. The F3 generation was used for the experiment. Virgin WT mtDNA *fog-2* L4 females (n = 400) and males (WT mtDNA n = 40, *Δnd-4* n = 40) were isolated onto separate NGM agar plate seeded with *E. coli* OP50 24 hours before the start of the mating experiment. Ten males were used for each of the Δ*nd-4 fog-2* lines (A-D). This is to ensure that the females have not been mated with

or fertilized prior to being paired with a male and that males retain all their sperm. Because we aimed to measure how the Δ*nd-4* mutation effects male mating ability, all the females used in this assay were WT mtDNA *fog-2*. After 24 hours, a single male and five females were transferred to a 35mm NGM agar plate with an *E. coli* OP50 bacterial lawn. The worms were allowed to mate for eight hours and then the females were isolated to individual plates. The next day the females were checked for fertilization. If there were eggs or offspring on the plate or if the female had fertilized eggs in her uterus, then the female was marked as fertilized.

3.3 Results

3.3.1. Δnd-4 does not impact survivorship in a fog-2 nuclear background nor the sex ratio

To test for survivorship, we established 24 35mm NGM agar plates seeded with OP50 *E. coli* each hosting ten L1 *C. elegans* (one Δ*nd-4* plate only had eight L1s). Twelve plates were for WT mtDNA *fog-2* individuals and the other 12 were for Δ*nd-4*-bearing worms (N_{fog-2} = 120, N_{nd-4} = 118). Of the 118 mutant individuals and 120 WT individuals, only 110 (93%) and 118 (98%) worms reached adulthood, respectively. We found that there was no significant difference in survivorship to adulthood (**Fig III-1**; Wilcoxon Rank sum test, z = -1.41, p = 0.15). The lack of statistical significance could reflect reduced power due to limited replication. To determine any alterations to the sex ratio, we established the sex of individual worms when they reached the L4 larval stage. After a chi-square analysis, we observed no change in the sex ratio in the mutant worms (**Table III-1**; X^2 = 0.145, *d.f.* = 1, p = 0.702).

3.3.2. Δnd-4-bearing females have reduced longevity

To examine possible differences in lifespan between the two sexes, we observed isolated males and females starting from the L4 larval stage until their death. In general, longevity for individuals with the Δ*nd-4* mutation significantly decreased by ~11% (**Fig. III-1**; Wilcoxon

Rank Sum Test z = -2.18, p-value = 0.03). An ANOVA analysis revealed that there was a significant difference between the sex and the number of days an individual was alive (**Fig III-2**; F_3-value = 6.24, $p < 0.001$). Surprisingly, we found that mutant females suffered a significant longevity cost when compared to the WT mtDNA *fog-2* males (Tukey HSD, p-value = 0.015, 95% C.I. = [0.35-4.57]) and females (Tukey HSD, $p < 0.001$, 95% C.I. = [-5.24 - -1.01]) and mutant males (Tukey HSD, $p = 0.002$, 95% C.I. = [0.87-5.22]). On average, Δ*nd-4* bearing females and males lived for 9.26 and 12.3 days, respectively. The lifespan of WT mtDNA females and males was 12.4 and 11.72 days, respectively. Note that longevity was calculated as number of days individuals lived past isolation at the L4 stage; it does not include how many days it took for the worms to develop from the L1 to L4 larval stage.

3.3.3. Productivity is reduced in crosses involving Δnd-4-bearing individuals

Four crosses (*fog-2* female x *fog-2* males (WT mtDNA control), Δ*nd-4* female x Δ*nd-4* males (mutant mtDNA control), Δ*nd-4* female x *fog-2* males, and Δ*nd-4* female x *fog-2* males) were made to quantify how the Δ*nd-4* mitotype influenced productivity. Using an ANOVA analysis, we found that there was a significant decrease in the number of offspring produced over the course of eight days (F-value = 12.54, $p < 0.0001$). Further analysis revealed that, in general, if a cross involved a Δ*nd-4*-bearing individual (regardless of sex), then ~62% fewer offspring were produced (**Fig III-1**; Wilcoxon Rank Sum Test, z = -5.00, $p < 0.0001$). When comparing the crosses involving mutant individuals (*fog-2* female x Δ*nd-4* males, and Δ*nd-4* female x *fog-2* males), there was no significant difference in the fertility between the sexes (**Fig III-3A**; Tukey HSD, $p = 0.60$, 95% C.I. = [-106.48-297.08]). While not significantly different from each other (**Fig III-3B**), Δ*nd-4* female x *fog-2* males crosses produced fewer progeny on average (189.6 offspring) when compared to the Δ*nd-4* female x Δ*nd-4* males (215.65 offspring), *fog-2* female x

Δ*nd-4* males (284.9 offspring) crosses. The WT mtDNA *fog-2* control crosses produced an average of 606.5 offspring (**Fig III-3B**).

3.3.4 Δnd-4 males are not less successful at fertilizing females

The male mating ability (MMA) assay was conducted to determine the mating success of Δ*nd-4* males. For every male (WT mtDNA *fog-2* or Δ*nd-4*), five virgin WT mtDNA *fog-2* females were plated and allowed to move around the plate for eight hours. Next the females were isolated to their own plates and checked for the presence of larval progeny or eggs the next day. We found that Δ*nd-4*- bearing males were not significantly less successful at fertilizing females relative to WT males (**Fig III-4**; Student's: $t = 1.32$, $p = 0.19$). An ANOVA analysis found there was significant difference between mutant lines (F-value = 3.25, $d.f. = 3$, $p = 0.033$) and *post hoc* analysis revealed that males from the B line did significantly worse than A males (Tukey HSD, $p = 0.033$, 95% C.I. = [-4.57- -0.14]), but not significantly different from males from lines C (Tukey HSD, $p = 0.16$, 95% C.I. = [-0.45- 3.85]) and D (Tukey HSD, $p = 0.1$, 95% C.I. = [-0.25- 4.05]). Lines A, C, and D were not significantly different from each other.

3.4 Discussion

Sexual reproduction is very complex. An important aspect to the evolution of eukaryotic life, sex is universal and creates important genetic dynamics that have puzzled scientists for decades (Smith, 1971, 1978). Sex is a risky endeavor in that it can lead to sexual antagonism and genomic conflict within and among species (Connallon and Clark, 2014; Havird *et al*., 2019). Genomic conflict can be seen in a variety of ways; here we focus on mito-nuclear conflict and how it can create sexual antagonism. The basis of this idea comes from the uniparental inheritance of the mitochondria in eukaryotes. Asymmetric inheritance allows for the possibility of one sex to suffer more consequences due to deleterious mutations that arise because selection

is only acting on the sex that is responsible for transmission. In the case of mitochondria, most plants and animals pass on the organelle maternally, meaning that males are more likely to pay the consequences of detrimental mitochondrial mutations (Frank and Hurst, 1996). Sexual antagonism is a widely studied topic, but *C. elegans* are an underutilized system in the context of mito-nuclear conflict and reproductive manipulation (Havird *et al*., 2019). However, sexual antagonism in aging has been well established to occur in the species (Tower, 2015). Here, we explore possible sex-specific effects due to a selfish mitotype, Δ*nd-4* (see Chapter II). This mitotype contains a 1034 bp deletion spanning the 3' end of the *cox-3*, a tRNA-*thr*, and the 5' end of the *nd-4* gene, as well as a nonsynonymous point mutation in *nd4L*.

One hypothesis that attempts to explain sexual antagonism created by mitochondrial variants is called the Mother's Curse, which posits that mutations that are neutral or nearly neutral in females but deleterious in males (weak form) can become fixed or rise to high frequency in a population due to genetic drift. Alternatively, mutations that are highly harmful to males, but beneficial to females can rise to fixation or high frequency because of selection acting on them (strong form; Havird *et al*., 2019). Only a few species have provided evidence for the Mother's Curse hypothesis (Vaught and Dowling, 2018) and it is considered rather controversial (Dowling and Adrian, 2019). This study tests the Mother's Curse in *fog-2 C. elegans*. While *C. elegans* are typically hermaphroditic, *fog-2* individuals are obligately outcrossing. This allows us to differentiate sex-specific consequences due to the Δ*nd-4* mitotype.

We performed three fitness assays (survivorship/sex ratio, longevity, and productivity) directly comparing males and females to each other. Overall, we found no difference in survivorship, but we did find a significant decrease in longevity and productivity (**Fig III-1**). The sex ratio was not altered due to mutant mitotype (**Table III-1**). This is not surprising since sex

ratio skew due to mitochondrial mutations have yet to be documented in animals (Havird *et al*., 2019). Interestingly, it was Δ*nd-4* bearing females that had significantly shorter lifespans than Δ*nd-4* bearing males and WT mtDNA *fog-2* individuals (**Fig III-2**). Moreover, aging studies in *C. elegans* have shown that males are the longer-lived sex due to pheromone (ascaroside) secretion (McCulloch and Gems, 2003; Maures *et al*., 2014). Because males secrete ascaroside throughout development (Kaplan *et al*., 2011; Izrayelit *et al*., 2012) and the worms used for the longevity assay were isolated at the L4 stage, it is possible that the significant decrease in Δ*nd-4* females was due to exposure to ascarosides before adulthood. However, considering that there was no difference between the WT mtDNA *fog-2* males and females, it could be that the reduced Δ*nd-4* female lifespan is due to a combination of pheromones and the detrimental mitotype or just the exposure to ascarosides. Alternatively, while ascarosides are harmful to hermaphrodites, males' lifespans are dependent on the number of males found in a population. Simply put, when males are surrounded by other males, the secreted ascarosides can significantly shorten their lifespan (Shi *et al*., 2017). Because individuals were isolated at the L4 larval stage, the longevity results suggest that the Δ*nd-4* mitotype more negatively effects females. Furthermore, it is possible that there was a difference between the sexes in the intracellular frequency of the Δ*nd-4* mitotype. The productivity assay consisted of four crosses: *fog-2* female x *fog-2* males (WT mtDNA control), Δ*nd-4* female x Δ*nd-4* males (mutant mtDNA control), Δ*nd-4* female x *fog-2* males, and Δ*nd-4* female x *fog-2* males. We found that any cross involving Δ*nd-4* individuals saw a significant decrease in productivity, but there was no significant difference among Δ*nd-4* crosses (**Fig III-3A**). While not significant, Δ*nd-4* female x *fog-2* males crosses produced the least number of offspring on average (**Fig III-3B**). Given that there is either no conclusive evidence (longevity) or no difference between Δ*nd-4* males and females in survivorship and

productivity, we do not find evidence to support the Mother's Curse hypothesis in the context of this mutant mitotype in *C. elegans*.

C. elegans males are notoriously inefficient at mating (Chasnov and Chow, 2002), so we next explored how Δ*nd-4* impacted male mating ability (MMA). We paired single virgin males with five virgin females and allowed them to mate for eight hours. After isolating the females and observing which ones were successfully fertilized, we found that the Δ*nd-4* mitotype has no significant effect on fertilization success (**Fig III-4**). We did observe that Δ*nd-4* line B had the fewest successful fertilization events. This might be due to differences between lines in the intracellular frequency of Δ*nd-4*. It is also possible that there are slight differences in the nuclear genetic background of the lines since they are from independent crosses between the original MA lines and the WT mtDNA *fog-2* controls. Although the crosses were designed to minimize the number of nuclear mutations from the original MA line that could be transferred to the *fog-2* nuclear background, it is conceivable that some of the original MA line nuclear mutations were nonetheless crossed into the *fog-2* background along with the Δ*nd-4* mitotype.

Overall, our results indicate that there are no sex-specific fitness effects due to the Δ*nd-4* mitotype. Sex-specific consequences from mitochondrial mutations are found in plants (Schnable and Wise, 1998) and animals (Beekman *et al*., 2014). There could be some explanations for the lack of sex-specific consequences. First, male-specific genes not associated with sperm production are highly conserved in *C. elegans*, so it is possible that there is no male-specific mito-nuclear conflict (Cutter and Ward, 2005). Second, males play an important role in maintaining genomic integrity by reducing inbreeding depression, and selection will may be strong for males with efficient mating ability (Anderson *et al*., 2010; Chasnov, 2013). It is important to note that despite selection for efficient males, selection acts very weakly on males in

general because they are so rare in nature (Chasnov, 2013). Other *Caenorhabditis* species have shown mito-nuclear interactions (Zhu *et al*., 2015; Wernick *et al*., 2019) and mito-nuclear epistasis (Chang *et al*., 2016). This leaves room for the possibility of more inquiry into sexual antagonism due to variant mitochondria.

3.5 Tables

Table III-1: Chi-square analysis of the sex ratio in *C. elegans* lines comparing Δ*nd-4* (N_{total} = 110) to WT (N_{total} = 118). X^2 = 0.145, *d.f.* = 1, p = 0.702

Line	Observed Sex (M/F)	Expected Sex (M/F)	Total	X^2 Value
fog-2	59/59	59/59	118	0
Δ*nd-4*	53/57	55/55	110	0.145

3.6 Figures

Figure III-1: Comparison of the mean relative fitness between *fog-2* (orange) and Δ*nd-4* (green) lines. All individuals used in the longevity (N_{fog-2} = 120, N_{nd-4} = 120), survivorship assays (N_{fog-2} = 120, N_{nd-4} = 118) and offspring from productivity crosses (*fog-2* female x *fog-2* males (WT mtDNA control), Δ*nd-4* female x Δ*nd-4* males (mutant mtDNA control), *fog-2* female x Δ*nd-4* males, and Δ*nd-4* female x *fog-2* males) were used in calculating the mean relative fitness. *p < 0.05, ****p < 0.0001, n.s. = not significant

Figure III-2: Boxplot comparing the longevity of males (blue) and females (red) within and among lines (WT mtDNA *fog-2* and Δ*nd-4*). Individual longevity was determined from time of isolation (L4 larval stage) to the time of death. The black lines in each boxplot represents the median lifespan of the individuals in each group. There was no significant difference in longevity between the Δ*nd-4* males and WT mtDNA *fog-2* males and females. Mutant females' longevity significantly decreased compared to Δ*nd-4* males (p = 0.002) and WT mtDNA *fog-2* males (p = 0.015) and females (p < 0.001).

Figure III-3A: The number of offspring produced after eight days between different crosses: *fog-2* male x *fog-2* female (WT mtDNA control, dark grey), Δ*nd-4* male x Δ*nd-4* female (mutant mtDNA control, light grey), Δ*nd-4* female x *fog-2* male (red), and Δ*nd-4* male x *fog-2* female (blue). The black lines in each boxplot indicate the median number of offspring per group. Note for each cross, two males and one female were paired. The Δ*nd-4* mitotype lowered productivity significantly for all crosses that involved a Δ*nd-4* bearing worm (p < 0.0001), but there were no significant sex-specific consequences (p = 0.60).

Figure III-3B: Bar graph of the average productivity of the different cross types: *fog-2* male x *fog-2* female (WT mtDNA control, dark grey), Δ*nd-4* male x Δ*nd-4* female (mutant mtDNA

control, light grey), Δ*nd-4* female x *fog-2* male (red), and Δ*nd-4* male x *fog-2* female (blue). The averages were taken from the total number of offspring produced per replicate per cross.

Figure III-4: Comparison of *fog-2* males (dark grey) and four lines (A[purple], B[green], C[brown], D[dark yellow]) of Δ*nd-4* bearing males mating ability. Every male (N_{fog-2} = 40, N_{nd-4} = 40) was paired with five *fog-2* females (N = 400) and allowed to mate for eight hours. Females were determined as fertilized the next day upon the observation of offspring or eggs present. There was no difference in the success of fertilizing females between the WT mtDNA and Δ*nd-4* worms. $p = 0.19$

Figure III-1: Comparison of the mean relative fitness between *fog-2* (orange) and Δ*nd-4* (green) lines.

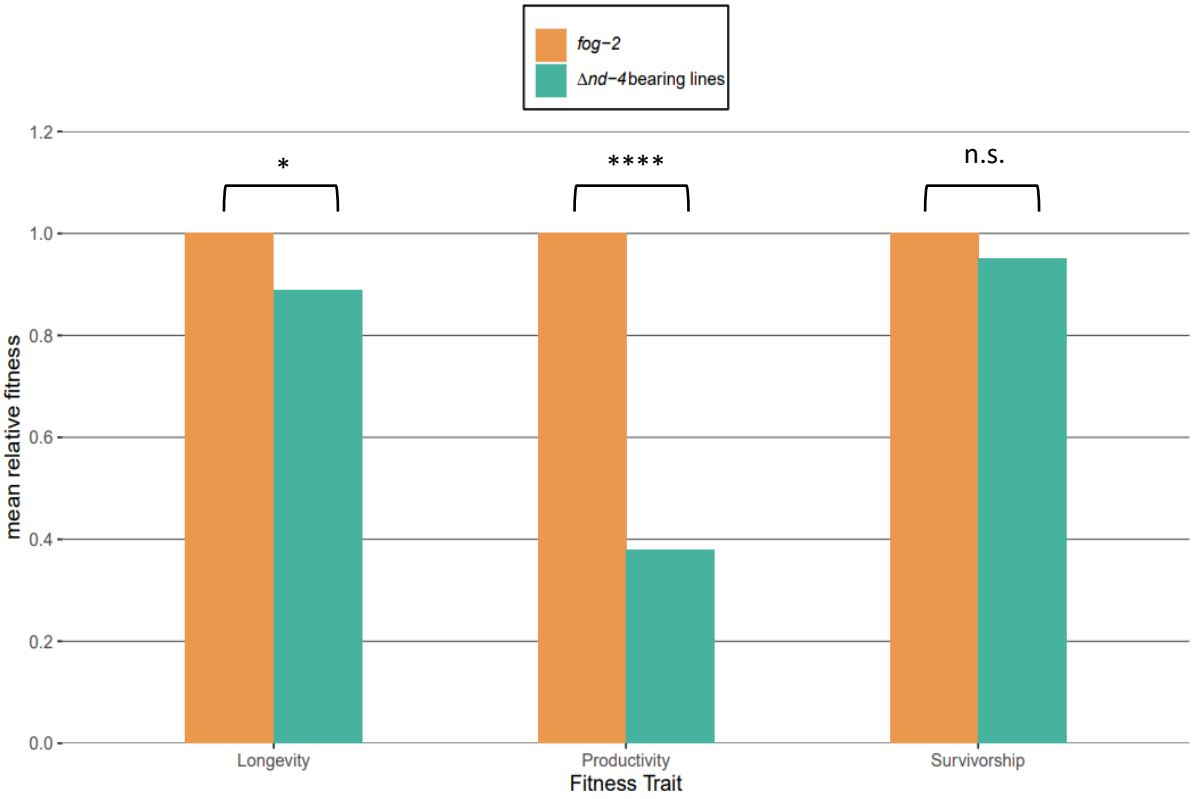


Figure III-2: Boxplot comparing the longevity of males (blue) and females (red) within and among lines (*fog-2* and Δ*nd-4*).

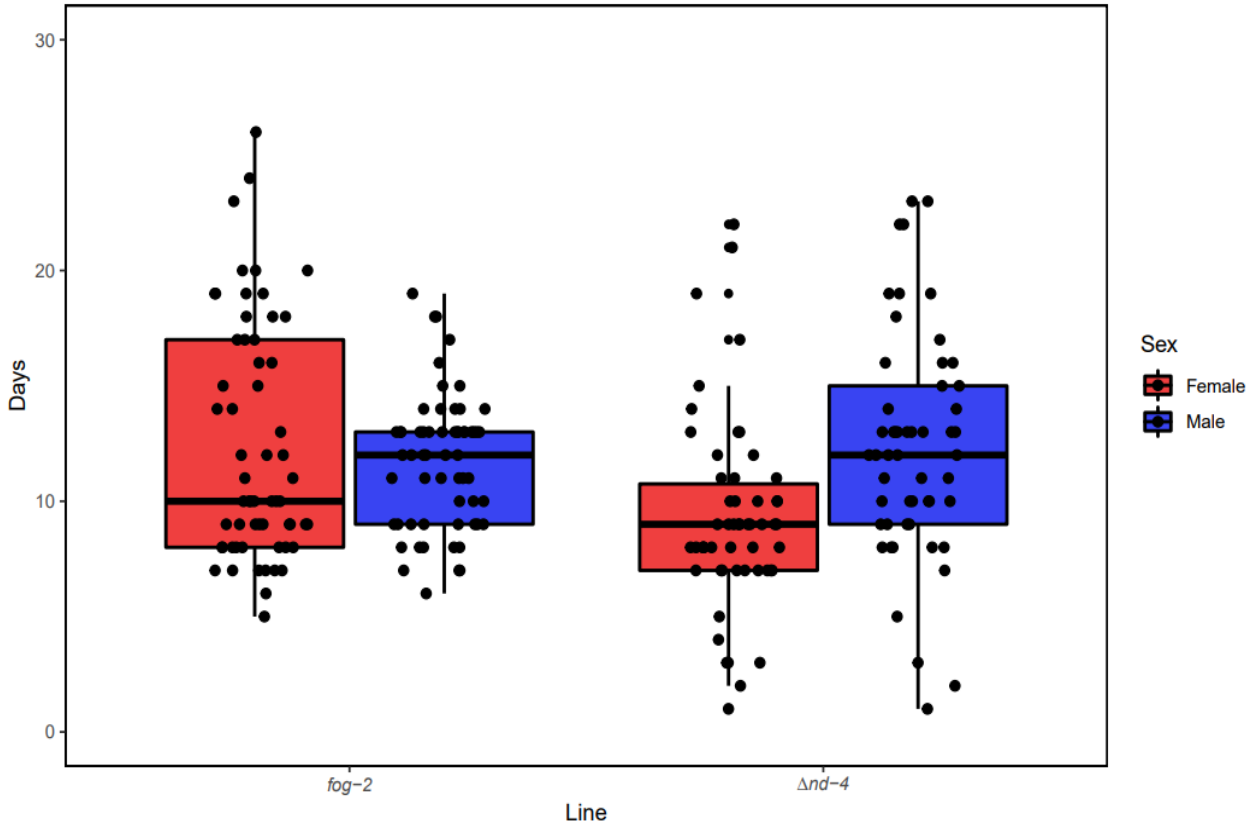


Figure III-3A: The number of offspring produced after eight days between different crosses: *fog-2* male x *fog-2* female (WT mtDNA control, dark grey), Δ*nd-4* male x Δ*nd-4* female (mutant mtDNA control, light grey), Δ*nd-4* female x *fog-2* male (red), and Δ*nd-4* male x *fog-2* female (blue).

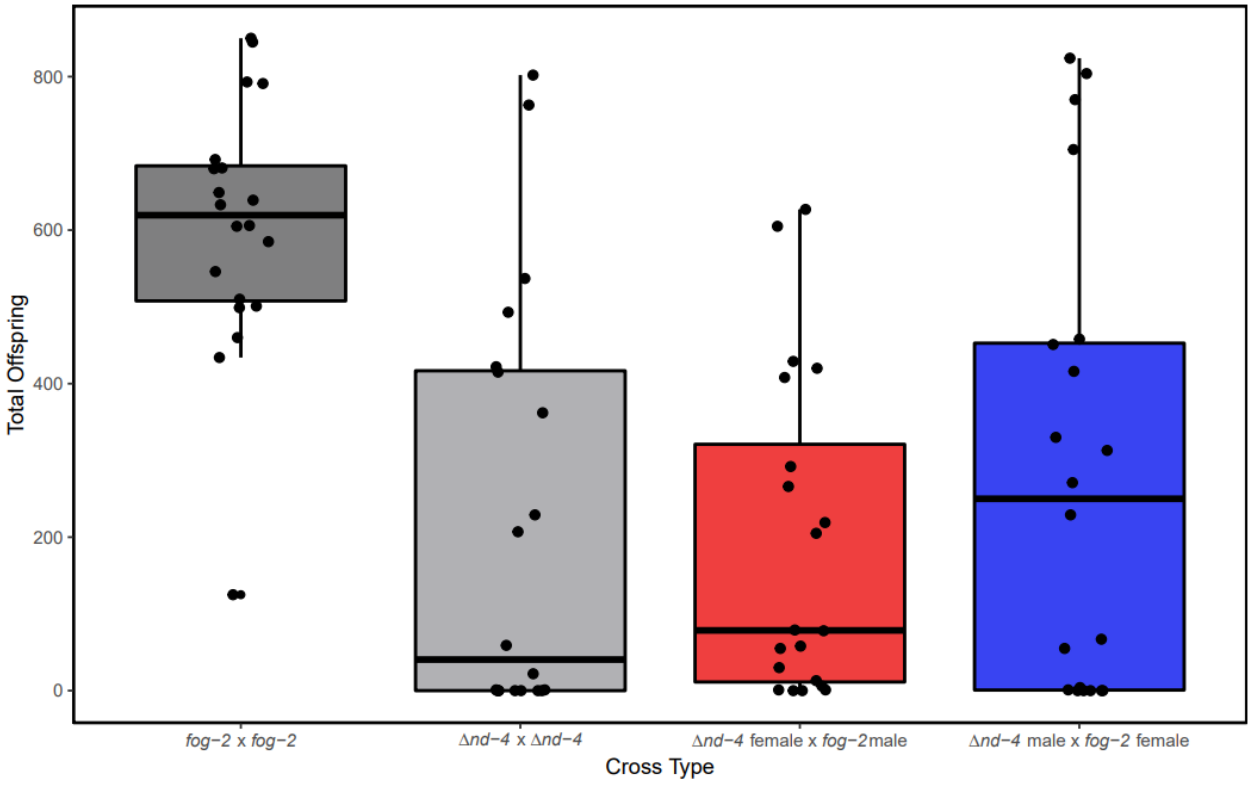


Figure III-3B: Bar graph of the average productivity of the different cross types: *fog-2* male x *fog-2* female (WT mtDNA control, dark grey), Δ*nd-4* male x Δ*nd-4* female (mutant mtDNA control, light grey), Δ*nd-4* female x *fog-2* male (red), and Δ*nd-4* male x *fog-2* female (blue).

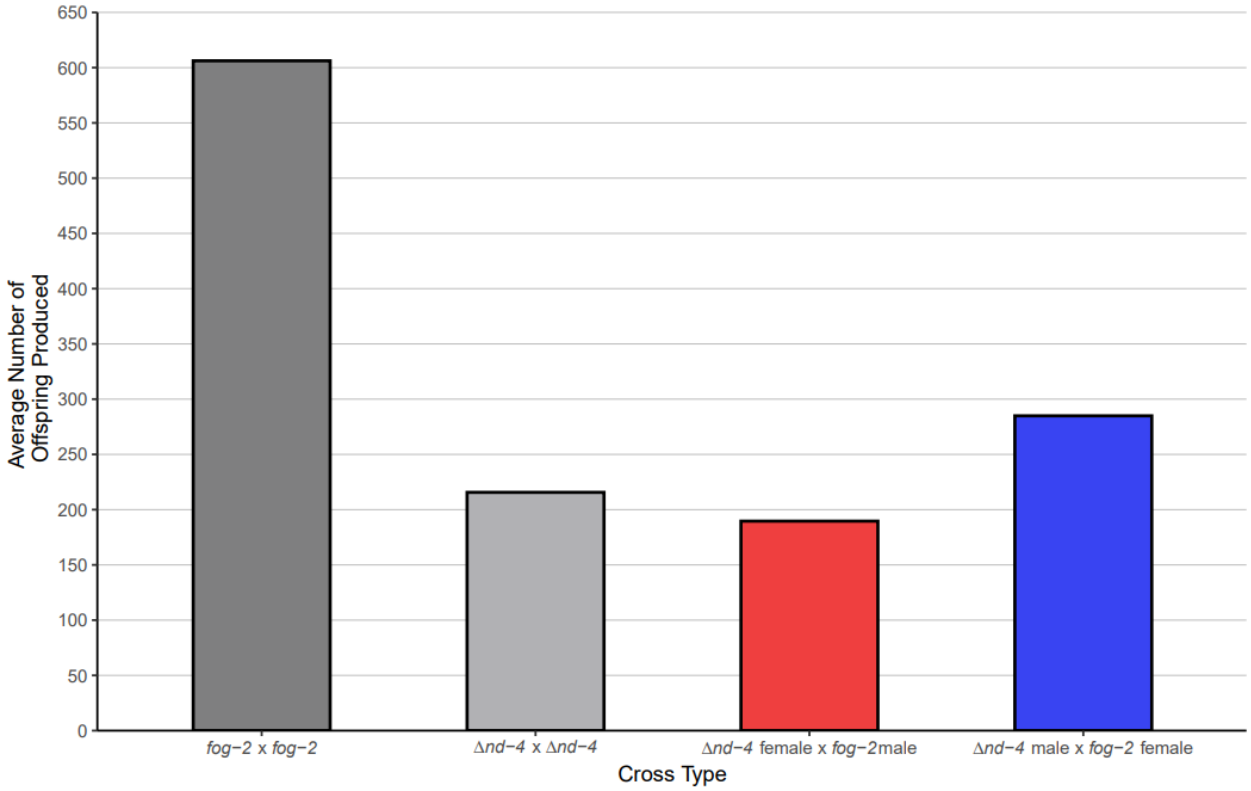
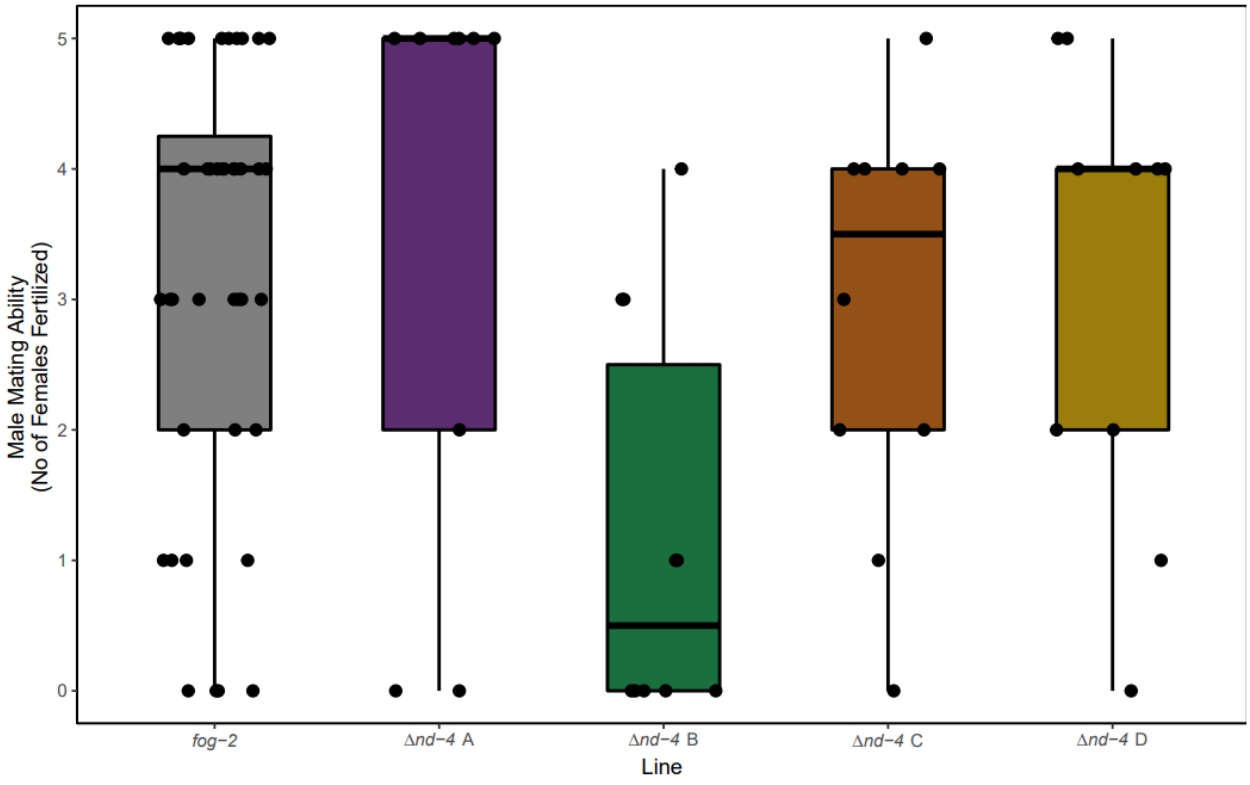


Figure III-4: Comparison of *fog-2* males (dark grey) and four lines (A[purple], B[green], C[brown], D[dark yellow]) of Δ*nd-4* bearing males mating ability.



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CONCLUSIONS

Mito-nuclear interactions are incredibly important to the evolution of eukaryotic life. Heteroplasmies are found very frequently ranging from low to high frequencies causing mito-nuclear disruptions and leading to disease (Parakatselaki and Ladoukakis, 2021). Heteroplasmies that rise to high frequencies and have neutral or deleterious fitness effects are considered selfish mitochondria and can have different evolutionary effects on species (Hurst and Werren, 2001). For example, selfish mitotypes can persist long enough for nuclear compensatory mutations to arise and showcase the coevolutionary process between the nuclear and mitochondrial genomes that have carried eukaryotes through evolutionary history (Zhu *et al*., 2015; Wernick *et al*., 2019). There could also be sex-specific effects due to aberrant mitochondria and exacerbate sexual antagonism in species (Connallon and Clark, 2014). In this thesis, we explore these very topics utilizing a spontaneous deletion that arose in the mitochondrial genome of the nematode *C. elegans*.

The Δ*nd-4* mitotype contains a 1034 bp deletion spanning the 3' end of *cox-3*, a tRNA-*thr*, and the 5'end of *nd-4*, and a nonsynonymous point mutation in *nd4L*. The deletion rose >80% and the substitution rose >90% during a previous mutation accumulation experiment in which *fog-2* male-female sib pairs were bottlenecked for <35 generations (Katju *et al*., 2022).

In Chapter II, we sought to confirm whether Δ*nd-4* was indeed selfish. Utilizing four life-history trait assays (development rate, productivity, survivorship to adulthood, and longevity), we found that there was a significant fitness decrease in all four traits for individuals bearing the Δ*nd-4* mitotype. Productivity decreased the most out of the life-history traits (63.7%). When competing with WT *C. elegans*, *Δnd-4* bearing worms rapidly went extinct in large populations. However, when not competing with WT worms, Δ*nd-4* bearing individuals were maintained at

large population sizes for several generations. Interestingly, most of the uncompeted populations saw a drastic decrease in the intra-individual Δ*nd-4* mitotype frequency after 60 generations, with some individuals fully losing the mutant mitotype and one line maintaining the Δ*nd-4* mitotype at ~90%. Lastly, we tracked the proliferation of Δ*nd-4* from a starting frequency of 34% over a bottlenecking regime for ten generations and found a rapid increase in the mitotype frequency. This result paired with the decrease in fitness confirmed that the Δ*nd-4* mutant mitotype was, indeed, selfish.

In Chapter III, we examined possible sex-specific effects due to a selfish mitochondrion. For these experiments, we used Δ*nd-4* bearing individuals with a *fog-2* nuclear background along with WT mtDNA *fog-2* nematodes. We tested three life-history traits (survivorship/sex ratio, longevity, and productivity), comparing males to females. We found no sex ratio skew, that Δ*nd-4* bearing females had significantly shorter lifespans, and Δ*nd-4* bearing individuals all had significantly lowered productivity. Because *C. elegans* females/hermaphrodites live shorter lives, it is difficult to tell in the Δ*nd-4* female longevity decrease is truly due to Δ*nd-4* or not (Ancell and and Pires-daSilva, 2017). When looking at how male mating ability is affected, we find no difference in mating success between WT mtDNA *fog-2* and Δ*nd-4* individuals.

This thesis builds on methods used to test for selfishness as well as expand upon ideas that have been underutilized in *C. elegans* (Gitschlag *et al*., 2016; Havird *et al*., 2019; Dubie *et al*., 2020). The number of selfish mitochondria in *C. elegans* is growing and with that so is the need to understand the inter- and intra-cellular dynamics of them. While that is not the focus of these studies, we do capitalize on the little research that has gone into sex-specific effects of mitochondrial mutations on *C. elegans*.

The interaction between harmful mitochondria and sexual antagonism is important, not only for evolutionary study, but also for mitochondrial disease research. It is well established that in systems which the mitochondria are strictly maternally inherited, males are more likely to suffer from detrimental mitotypes (Frank and Hurst, 1996). There are not many studies on the possible sexual antagonism brought on by mitochondrial mutations in *C. elegans*. However, this model system is incredibly useful to exploring Mother's Curse or other sexual antagonistic dynamics because *C. elegans* have multiple breeding systems. This will allow scientists to explore more deeply sexual antagonism not only in a dioecious line, but also in a hermaphroditic line as well. Sexual antagonism in hermaphrodites is less obvious. However, in *C. elegans*, male-specific genes are highly conserved (Cutter and Ward, 2005). A useful way of elucidating sex-specific effects of mitochondrial heteroplasmies in hermaphrodites is to look at the gene expression in male-specific and female-specific genes and compare that to compensatory mutations that might arise in the sex-specific genes.

Sperm and oocyte studies would also be useful in determining sex-specific outcomes of mitochondrial mutations. Of the fitness consequences that were discussed in Chapters II and III, *C. elegans* fertility was the most effected life-history trait. Furthermore, there were slight differences between males and females in sex-specific effects. It could be possible that there was a difference in sperm and oocyte quality. The mitochondria play a major role in the quality of oocytes (Cummins, 2002) and mitochondrial dysfunction increases with age (Quesada-Candela *et al*., 2021). On the other hand, *C. elegans* sperm triggers proteostasis enhancement when prepping the oocyte for fertilization. Dysfunctional mitochondria in the sperm could play a role in disrupting this interaction and cause lowered fertility (Bohnert and Kenyon, 2017). In the context of studying sex-specific effects of mitochondrial mutations in hermaphrodites, *C.*

elegans is a useful model system due to the ease of experimental manipulation. In a broader sense, there is still not much known about the mechanisms of mitochondrial dysfunction and sexual antagonism. Examining related questions will give further insight on how mitochondrial diseases spread as well as illustrate aspects of the evolution of sex that are still puzzling scientists.

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