MATING AT ADVANCED AGE: HOW OLD NEMATODES MODULATE PHEROMONE PRODUCTION TO ATTRACT YOUNG MALES

Thesis by

Daniel Leighton

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2016

(Defended April 21, 2016)

Acknowledgements

Before I discuss my research, I would like to dedicate this thesis to the people who helped me reach this point in my life and my career.

First and foremost, I must thank my wife, Carrie, for her patience, her love, and her cheerleading these past six years.

Next, I obviously have to thank my advisor, Paul Sternberg, for giving me a laboratory to call home, and for always having supporting essentially every crazy idea I came up with. Having gotten my bachelor's degree at Caltech as well, I actually had the opportunity to take Paul's genetics class before even applying to graduate school, and I also read some of his early papers. The one thing I remember thinking the most was, "Wow, how weird do you have to be to get this fascinated by worm vulvas?" But when I was thinking about which laboratories to rotate in during my first year, Paul came to mind. I wasn't sure I wanted to study nematodes at all, but Paul stuck out in my memory as one of the Caltech professors who made science seem fun and exciting. I thought I might have a good time in his lab, and I was right. Before long, *I* was fascinated by worm vulvas as well.

I would also like to thank the various members of the Sternberg lab who have helped me tremendously along this path. When I officially joined the lab, I wanted to develop a technique to more accurately measure population growth defects. Paul directed me to a fellow graduate student, Oren Schaedel, to get advice on carrying out this project. Oren told me to pick another project, which turned out to be a great idea! For that I thank him. But I did spend a whole two weeks playing with the idea, and during that time my lab roommate, Andrea Choe, suggested I help on one of her own projects to get some more practice working with worms. This turned out to be an amazing offer, as I quickly began making discoveries, and followed Oren's advice to

drop my original project. Andrea gave me her blessing to carry on her project after she graduated, and the rest is, well, chapter 2 of this thesis.

Over the years, I have had excellent collaborations with Professors Frank Schroeder at Cornell University, Asher Cutter at the University of Toronto, and Matthew Gronquist at SUNY Fredonia. Hopefully my work with them gets published soon.

I've also had the pleasure of supervising several excellent high school students and one undergraduate. I would like to particularly thank Ashley Hebard and Shannon Wu for their amazing focus and hard work.

The members of my thesis committee have provided invaluable guidance during my time as a graduate student.

Many other people have helped as well. Mihoko Kato and Hillel Schwartz have provided immense quantities of advice over the years. Whenever I had a problem that required common sense to solve, Chris Cronin was there. Ditto Igor Antoshechkin and Vijaya Kumar with sequencing, and Chris Grove for help with programming. Barbara Perry, Gladys Medina, Mary Alvarez, and Sarah Torres have been the helping hands behind the scenes, making sure the lab actually stays functioning. David Angeles has been an amazing collaborator, using bioinformatics to turn my mundane data into amazing stories. I must also thank Ryoji Shinya, Han Wang, Alli Akagi, Katie Brugman, James Lee, Ravi Nath, Pei-Yin Shih, Yen-Ping Hsueh, and the rest of the Sternberg lab members for their help and friendship over the last several years. I would like to thank Christine Chang, my high school best friend, for listening to my worm rants when everyone in the lab had already gone home. And lastly, I would like to thank John DeModena for making the lab such an interesting place to be even late at night.

Preface

From a young age, I dreamed of doing something to help the world. Starting when I was sixteen, that dream became a little more specific: I wanted to help the world *with science*. I can give the most thanks for that to two of my high school teachers, Miss Provost and Mr. Walsh.

When I took my first summer research job with Professor Yun Yen at the City of Hope in 2007, I knew I had made the right choice. In lower division courses, we looked up answers to questions in our textbooks, and that was OK. In upper division courses, the answers were in recently published papers, and that was fun. But in the lab, the answers to the professor's questions were not written down anywhere. We had to find out for ourselves, and that was exhilarating. For when you get that result, when you look at that data, from then until you share it, you are the only person in the world who knows the answer to that question. It got me hooked on research, and I haven't once looked back.

Over time my motivations have changed. I still hope my work winds up helping the world, but now I'm driven by the sheer joy of learning.

I used to think of nematodes as just these dumb little worms that lived in the dirt, whose only functions were to eat, mate, and find a place to do the first two things. But upon really diving into this field, I've seen that my old perspective missed so much. Although the nematode's nervous system is so simple people argue over whether the largest nerve cluster can even be called a brain, we still don't understand how it works! A system of just a few hundred neurons and a few thousand synapses has proven so complicated, we can only get at understanding it one bit at a time. And the nematode's response to environmental signals? Immensely complicated! So too are the nematodes' olfactory signaling amongst one another. These little worms are having chemical conversations, and making life-or-death decisions about where to go

and what to eat, right under our feet, and we have only the faintest idea how any of it works. I think I might be happy spending the rest of my life trying to make sense of it.

These worms are talking to each other, and I would like to know what they're saying. And that brings me to my thesis.

Abstract

Nematodes have been studied for centuries in their roles as pathogens of humans, crops, and livestock. In more recent times, the free-living nematode *Caenorhabditis elegans* and its close relatives have been heavily studied as genetic and developmental model organisms. Despite the extent of research into nematode biology and lifestyle, relatively little is known about communication between nematodes. In the last decade, there has been a burst of research into identifying the pheromones secreted by nematodes, as well as determining their effect on other nematodes in the population.

The bulk of pheromone research has focused on the chemical identification of olfactory signals, and studying the behavioral and physiological responses of worms exposed to these signals. We report the discovery of a new *C. elegans* mating pheromone, and an attempt to dissect the pathway that regulates its production. Instead of studying what a worm "hears" when this signal is received, we hope to understand what the worm that produces the signal is trying to "say".

We also review the existing literature on nematode mating pheromones, highlighting the most stunning recent discoveries, and point out several questionable claims frequently made by authors in the field.

Table of Contents

Chapter 1:

Mating pheromones of *Nematoda*: Olfactory signaling with physiological consequences

Abstract

Secreted pheromones have long been known to influence mating in the phylum *Nematoda*. The study of nematode sexual behavior has greatly benefited in the last decade from the genetic and neurobiological tools available for the model nematode *Caenorhabditis elegans*, as well as from the chemical identification of many pheromones secreted by this species. The discovery that nematodes can influence one another's physiological development and stress responsiveness through the sharing of pheromones, in addition to simply triggering sexual attraction, is particularly striking. Here we review recent research on nematode mating pheromones, which has been conducted predominantly on *C. elegans*, but there are beginning to be parallel studies in other species.

Introduction

Nematoda is a diverse phylum of worms that occupy a variety of terrestrial, aquatic and marine habitats. Most nematode species are gonochoristic, consisting of males and females that must locate one another and mate to reproduce. Research on nematode mating pheromones began in the 1960s with an eye toward understanding the mating habits of animal- and plant-parasitic nematodes. Since then, it has been found that dozens of nematode species, most of them vision- and hearing-impaired by nature, locate one another by the reception of secreted molecules (reviewed in [1]).

Most research on nematodes is conducted in the small, transparent *Caenorhabditis elegans*, which lives on rotting fruit and utilizes insect vectors as phoretic hosts. This nematode, unlike most, is androdiecious in nature, consisting of self-fertilizing hermaphrodites and rare males (reviewed in [2]). The hermaphrodites are incapable of mating with one another, and may only self-fertilize using a limited supply of self-sperm generated during larval development –

reproduction after the exhaustion of self-sperm can only proceed by mating with a male. That mating is strictly unnecessary for survival of *C. elegans* makes this species an excellent model for the study of mating behavior [3], as the mating process can be disrupted at nearly every step without sacrificing the organism's viability [4].

The discovery that many disparate species utilize ascarosides, a nematode-specific family of glycolipids, for communication, has greatly accelerated the rate of research in this field (reviewed in [5]). The research reviewed here largely took advantage of the ability to identify specific pheromones and challenge worms with pure synthetic compounds to uncover the precise neurological and physiological activities of these molecules.

Ascarosides

The ascarosides are glycosides of the dideoxysugar ascarylose. These molecules always include a lipid tail, and may be conjugated to amino acid derivatives or other small molecules [6]. Ascarosides are now known to be produced by over twenty species of nematode from multiple clades, including free-living, vertebrate parasitic and insect parasitic worms [7]. Ascarosides have been demonstrated as mate-attracting pheromones both *C. elegans* [8] and *Panagrellus redivivus* [9], despite these species' significant evolutionary distance, lying in different families. Ascarosides have also been shown to promote entrance into dauer, an alternative non-feeding and stress resistant larval stage, in *C. elegans* [10], *Heterorhabditis bacteriophora* [11], and *Pristionchus pacificus* [12]. As would be expected of signaling molecules, ascaroside production varies with a worm's age and environment [13]. In both *C. elegans* and *P. redivivus*, the different genders secrete specific ascarosides in vastly different quantities, with each gender producing the attractants for the other. In addition, the *C. elegans* hermaphrodite secreted pheromones are repulsive to other hermaphrodites [9,14]. Taken together, this evidence suggests that

ascarosides are evolutionarily ancient signaling molecules that may serve as mating pheromones across much of *Nematoda*.

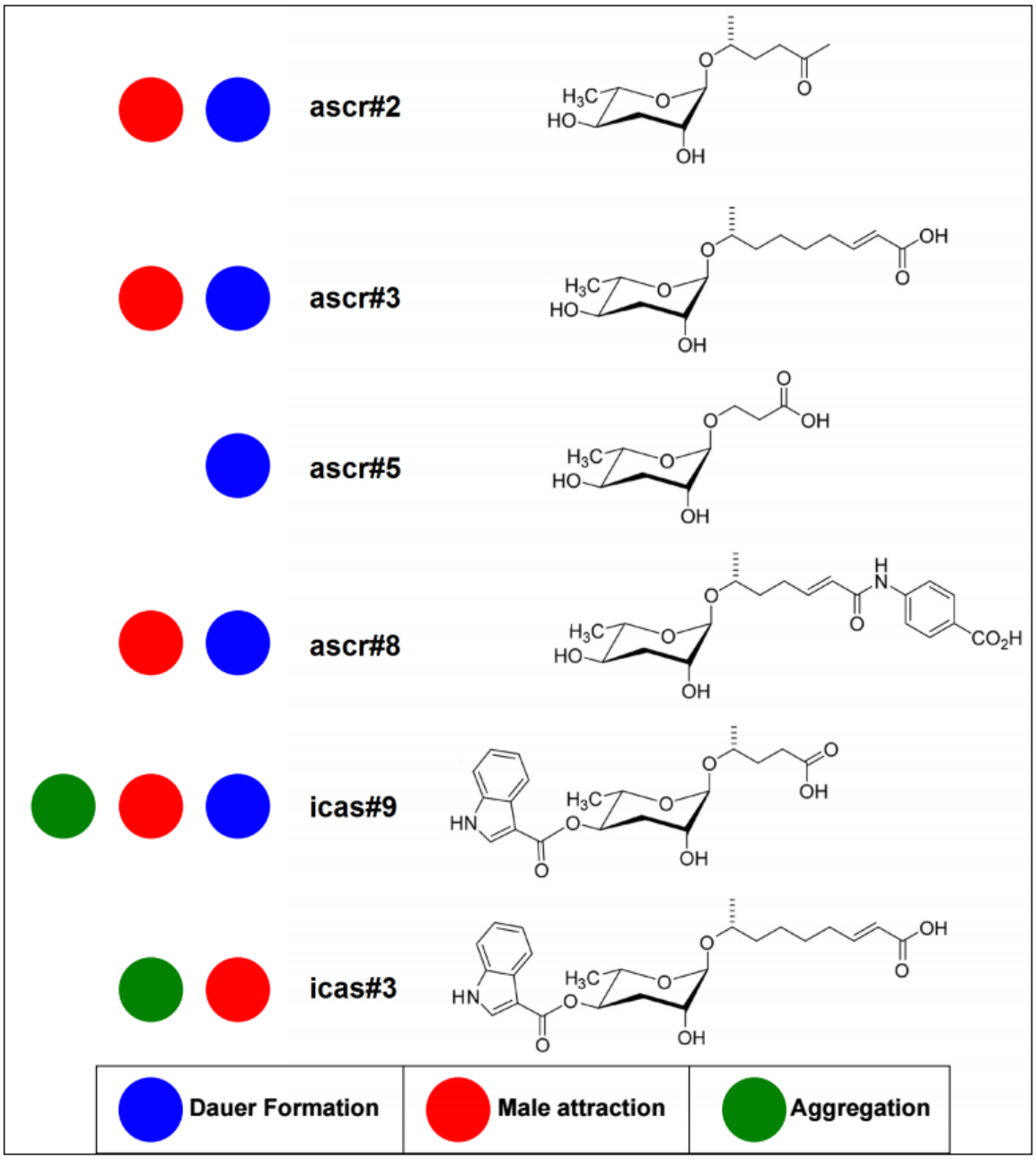

Figure 1: The behavioral response of *Caenorhabditis elegans* to specific ascarosides is highly dependent on chemical structure.

In addition to being a highly diverse molecular family with over 150 members, the specific activity of each ascaroside is highly dependent on its chemical structure [15] (Fig. 1), and the same ascaroside can have extremely different effects on different species. For example, ascr#1

is a potent mate-attracting pheromone in *P. redivivus*, but promotes dauer formation in *C. elegans* [9,10,16]. In fact, evolution of ascaroside signaling appears to be extremely rapid even within a species. The production and reception of dauer pheromones by both *C. elegans* and *P. pacificus* differs markedly amongst wild isolates of both species, possibly as a result of intense intraspecific competition over food resources [12,17]. Different strains of the same nematode are even known to mate at different rates, though this may have both pheromone-related and other reasons [18]. Ascarosides are also known to trigger feeding behavior in nematophagous fungi [19], and immune reactions in plant hosts [20], which likely apply additional pressure on the rapid evolution of nematode pheromones. Additionally, the ability of a nematode to locate a mate depends on more than simply chemotaxis to a pheromone cocktail. For example, Sammut et al. (2015) have recently shown that *C. elegans* males can be conditioned to associate a particular salt concentration with potential mates [21].

The essentially invariant anatomy of *C. elegans*, coupled with the ability to identify specific neurons under Nomarksi microscopy, has allowed researchers to identify pheromone-responsive neurons through laser ablation experiments. The ADL, ASI, and ASK sensory neurons are necessary for normal response to mating pheromones, and thus are expected to express mating pheromone receptors [22-24]. One exciting finding from these studies is that the male neuronal response to mating pheromone is exhibited within the hermaphrodite nervous system as well. However, the stereotypical male behavioral response is simultaneously inhibited by neurons that only react to mating pheromone in the hermaphrodite [24].

Currently, six ascaroside receptors have been identified in *C. elegans*: *srg-36*, *srg-37*, *srbc-64*, *srbc-66*, *daf-37*, and *daf-38*. Two of these receptors, *srg-36* and *srg-37*, have homologs with conserved function in *Caenorhabditis briggsae*. All six receptors are G-protein-coupled-receptors (GPCRs), consistent with findings that GPCR function is important for sensation of ascarosides

[25-28]. It has proven difficult, however, to determine precise relationships between pheromones and their receptors. It is not known whether ascaroside receptors function as single proteins, or as homo or heterodimers. Two yeast-based assays have recently been developed specifically for analysis of *C. elegans* GPCR activity [29,30]; this may allow for more rapid identification of ligand-receptor relationships than the mammalian and *Xenopus* based assays traditionally used for this purpose.

Hermaphrodite versus female behavior

There has been a common conception in nematode literature that *C. elegans* hermaphrodites have lost certain female traits through evolution (e.g., [31-33]). Traits claimed to have been lost by hermaphrodites include the production of volatile mate-attracting pheromone and mating-induced torpor (which may aid the male in locating the vulva). In fact, evidence for both of these traits may be found in *C. elegans*, but mating behavior in general is repressed by the presence of sperm. As with a recently mated female, a young hermaphrodite exhibits no production of volatile pheromone, but resumes production upon sperm depletion, whether due to mutation or age [34]. This change in hermaphrodite pheromone production is also observed in the plant parasitic nematode *Bursaphelenchus okinawaensis* [35].

Garcia et. al ([36]) argued that hermaphrodites and females differ primarily because of age, but it is worth revisiting the observations in light of the recent finding of sperm-regulated physiology in hermaphrodites. In particular, the ability of males to successfully insert their spicules into hermaphrodites or xenospecfic females was assayed. There appears to be an effect of both age and sperm status. Specifically, the authors investigated the number of mating attempts made by a male before successful spicule insertion, depending on the strain of hermaphrodites he was presented with. The data for this experiment shows a dramatic reduction in the number of

failed attempts if the hermaphrodite is spermless due to mutation, and a further reduction if the hermaphrodite is aged past the normal period of self-fertilization. These data indicate there are separate youth- and sperm-correlated inhibitory effects on male copulation. Similar results were reported by another group in the same year [37]. Young *C. elegans* pseudofemales may be more difficult to mate with than young true females of another species, but the difference is far smaller than that between a female and a young hermaphrodite. *C. elegans* males do not induce torpor in females of other species upon mating, but this may simply be due to divergence of signals or receptors. Keeping in mind the significant differences in mating behavior between isolates of the same species, we believe a proper test of this torpor hypothesis would be to challenge males from multiple *C. elegans* isolates with aged and feminized hermaphrodites, also of various isolates. While there are genuine differences between hermaphrodites and true females of closely related species, in many respects the hermaphrodites are simply behaving as sperm replete females.

Physiological effects of pheromone exposure

A major shift in the discussion of nematode mating pheromones has occurred with the recognition that they are responsible for more than just chemoattraction. An early paper that attempted to address this topic, Timmermeyer et al. [33], showed that *C. remanei* females that had received copulatory plugs from their mates were more fecund than their unplugged sisters, despite receiving a similar number of matings. This observation suggests that male-deposited mating plugs, which may contain signaling compounds, might act to promote egglaying, oocyte development, or some other fertility promoting process in the receiving female. However, the possibility remains that plugged females had simply received or retained more sperm than their unplugged sisters in these experiments.

Very recently, Aprison and Ruvinsky [38] showed that exposure to male pheromones has a substantial and beneficial effect on recovery from heat stress in both *C. elegans* and *C. remanei*[38]. Heat stress is known to reduce fertility in *C. elegans* hermaphrodites, to the point of sterility at sufficient extremes. Exposure to either males or male pheromones is sufficient to restore partial fertility. The authors focused on two ascaroside pheromones, ascr#3 and ascr#10. These pheromones appear to promote clearance of gonadal blockages and improved sperm guidance, respectively. These two pheromones are in fact produced by both genders, but in different proportions. A hermaphrodite-like synthetic cocktail does not promote recovery, though it is unknown how the hermaphrodite can exercise this level of discretion when the molecules are the same.

Not all pheromone exposure serves to promote fertility. Dauer pheromones are believed to alert nematodes to conditions of overcrowding. In young larvae, these pheromones cause entry into the long-lived dauer state, but in adult hermaphrodites, these same pheromones cause reduced production of sperm guidance signals, leading to lower rates of fertilization[39]. Both this effect and the heat-stress amelioration of male pheromones are dependent on the gene *daf-7*, which encodes a TGF-β ligand secreted by sensory neurons [38,39]. It seems that *C. elegans* and other nematodes have evolved to reproduce at variable rates depending on environmental and social conditions.

Battle of the sexes

It has been known for over 40 years that mating with a male reduces the lifespan of a nematode, first in *Panagrellus* [40], then in *C. elegans* [41], and more recently in *C. remanei* [42]. It has even been shown that in a mutant gonochoristic *C. elegans* strain raised for 100

generations, males have evolved to cause even more extreme reduction in lifespan, while the pseudofemales have evolved a resistance to this effect [43].

While the act of mating with a male causes damage to a worm's cuticle [44], this is not sufficient to reduce lifespan. Rather, the major impact on hermaphrodite or female lifespan appears to result from exposure to male pheromone and transfer of seminal fluid, and is mediated by hermaphrodite/female neuronal activity (especially insulin signaling). The shortening of the hermaphrodite lifespan is sometimes accompanied by substantial shrinking of the body and sensitization to osmotic stress prior to death [45,46]. It is frequently argued that the reduction in female/hermaphrodite lifespan is an example of the battle of the sexes – that males reduce the lifespan of their mate for selfish purposes. This argument typically appears in the absence of experiments to demonstrate that lifespan reduction is not coupled to hermaphrodite/female benefit.

While the fact that lifespan reduction can be achieved without fertilization, or even mating, is strong evidence against a "death through reproductive exhaustion" hypothesis, it does not rule out that the female is making physiological changes in anticipation of increased reproductive rate. This has been presented as a "life span versus reproduction" hypothesis. Settling this question is complicated by the strong influence of experimental design on the relationship between longevity and hermaphrodite fecundity. Wu et al. (2012) found that perpetual mating with five males per hermaphrodite yields a positive correlation between lifespan and fecundity, mostly due to lifespans becoming so short that hermaphrodites die before exiting from the fertile phase of life. In a much more mild setup with one male per hermaphrodite, with mating only lasting for 24 hours, there was a negative correlation between lifespan and fecundity [47]. Shi and Murphy ([45]) argue that, among other reasons, because *daf-12* mutants can extend their fertile period through mating without suffering a reduction in lifespan, lifespan shortening

is not linked to hermaphrodite benefit. However, the *daf-12* mutants used in this study suffer from shortened lifespan to begin with. A convincing proof that lifespan reduction is to the hermaphrodite's detriment would be a demonstration that pheromone-insensitive hermaphrodites can yield at least as many viable offspring after mating as wild-type hermaphrodites.

Conclusion

More than ten years ago, virtually all research on nematode mating pheromones was limited to merely demonstrating that they existed in various species, and motivated chemoattraction. Few pheromones were chemically identified. In the last ten years, we have seen an accelerated pace of research on nematode pheromones thanks to the discovery of ascarosides, whose use as pheromones appears to be conserved across much of *Nematoda*.

With the ability to now synthesize pure pheromones, we expect future research to uncover the neurological and biochemical activity of many pheromones that we may better understand how the worm sexes communicate in the wild. While the vast majority of research in this field has been conducted in the model organism *C. elegans*, the knowledge of the conservation of ascaroside pheromones, as well as the increased ease with which even non-model organisms can be genetically modified, should make it easier for future research to uncover similar systems in other nematodes.

References

1. Hsueh Y-P, Leighton DW, Sternberg P: **Nematode Communication**. In *Biocommunication of Animals*. Edited by Witzany G: Springer Netherlands; 2014:383-407.
2. Corsi AK, Wightman B, Chalfie M: **A Transparent window into biology: A primer on Caenorhabditis elegans**. In *WormBook*. Edited by Community TCeR: WormBook; 2015.
3. Hodgkin J: **Male Phenotypes and Mating Efficiency in CAENORHABDITIS ELEGANS**. *Genetics* 1983, **103**:43-64.
4. Liu KS, Sternberg PW: **Sensory regulation of male mating behavior in caenorhabditis elegans**. *Neuron* **14**:79-89.
5. Ludewig AH, Schroeder FC: **Ascaroside signaling in C. elegans**. In *WormBook*. Edited by Community TCeR: WormBook; 2013.
6. von Reuss SH, Bose N, Srinivasan J, Yim JJ, Judkins JC, Sternberg PW, Schroeder FC: **Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in C. elegans**. *J Am Chem Soc* 2012, **134**.
7. Choe A, von Reuss Stephan H, Kogan D, Gasser Robin B, Platzer Edward G, Schroeder Frank C, Sternberg Paul W: **Ascaroside Signaling Is Widely Conserved among Nematodes**. *Current biology : CB* 2012, **22**:772-780.
8. Pungaliya C, Srinivasan J, Fox BW, Malik RU, Ludewig AH, Sternberg PW, Schroeder FC: **A shortcut to identifying small molecule signals that regulate behavior and development in Caenorhabditis elegans**. *Proceedings of the National Academy of Sciences* 2009, **106**:7708-7713.
9. Choe A, Chuman T, von Reuss SH, Dossey AT, Yim JJ, Ajredini R, Kolawa AA, Kaplan F, Alborn HT, Teal PEA, et al.: **Sex-specific mating pheromones in the nematode Panagrellus redivivus**. *Proceedings of the National Academy of Sciences* 2012, **109**:20949-20954.
10. Srinivasan J, Kaplan F, Ajredini R, Zachariah C, Alborn HT, Teal PEA, Malik RU, Edison AS, Sternberg PW, Schroeder FC: **A blend of small molecules regulates both mating and development in *Caenorhabditis elegans***. *Nature* 2008, **454**:1115-1118.
11. Noguez JH, Conner ES, Zhou Y, Ciche TA, Ragains JR, Butcher RA: **A Novel Ascaroside Controls the Parasitic Life Cycle of the Entomopathogenic Nematode Heterorhabditis bacteriophora**. *ACS Chemical Biology* 2012.
12. Mayer MG, Sommer RJ: **Natural variation in Pristionchus pacificus dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones**. *Proceedings of the Royal Society of London B: Biological Sciences* 2011, **278**:2784-2790.
13. Kaplan F, Srinivasan J, Mahanti P, Ajredini R, Durak O, Nimalendran R, Sternberg PW, Teal PEA, Schroeder FC, Edison AS, et al.: **Ascaroside Expression in Caenorhabditis elegans Is Strongly Dependent on Diet and Developmental Stage**. *PLoS ONE* 2011, **6**:e17804.
14. Izrayelit Y, Srinivasan J, Campbell SL, Jo Y, von Reuss SH, Genoff MC, Sternberg PW, Schroeder FC: **Targeted Metabolomics Reveals a Male Pheromone and Sex-Specific Ascaroside Biosynthesis in Caenorhabditis elegans**. *ACS Chemical Biology* 2012, **7**:1321-1325.
15. Hollister KA, Conner ES, Zhang X, Spell M, Bernard GM, Patel P, de Carvalho ACGV, Butcher RA, Ragains JR: **Ascaroside activity in Caenorhabditis elegans is highly dependent on chemical structure**. *Bioorganic & Medicinal Chemistry* 2013, **21**:5754-5769.
16. Jeong P-Y, Jung M, Yim Y-H, Kim H, Park M, Hong E, Lee W, Kim YH, Kim K, Paik Y-K: **Chemical structure and biological activity of the Caenorhabditis elegans dauer-inducing pheromone**. *Nature* 2005, **433**:541-545.

17. Diaz SA, Brunet V, Lloyd-Jones GC, Spinner W, Wharam B, Viney M: **Diverse and potentially manipulative signalling with ascarosides in the model nematode C. elegans**. *BMC Evolutionary Biology* 2014, **14**:1-8.
18. Bahrami AK, Zhang Y: **When Females Produce Sperm: Genetics of C. elegans Hermaphrodite Reproductive Choice**. *G3: Genes|Genomes|Genetics* 2013, **3**:1851-1859.
19. Hsueh Y-P, Mahanti P, Schroeder Frank C, Sternberg Paul W: **Nematode-Trapping Fungi Eavesdrop on Nematode Pheromones**. *Current Biology* 2013, **23**:83-86.
20. Manosalva P, Manohar M, von Reuss SH, Chen S, Koch A, Kaplan F, Choe A, Micikas RJ, Wang X, Kogel K-H, et al.: **Conserved nematode signalling molecules elicit plant defenses and pathogen resistance**. *Nat Commun* 2015, **6**.
21. Sammut M, Cook SJ, Nguyen KCQ, Felton T, Hall DH, Emmons SW, Poole RJ, Barrios A: **Glia-derived neurons are required for sex-specific learning in C. elegans**. *Nature* 2015, **526**:385-390.
22. Jang H, Kim K, Neal SJ, Macosko E, Kim D, Butcher RA, Zeiger DM, Bargmann CI, Sengupta P: **Neuromodulatory state and sex specify alternative behaviors through antagonistic synaptic pathways in C. elegans**. *Neuron* 2012, **75**:585-592.
23. Macosko EZ, Pokala N, Feinberg EH, Chalasani SH, Butcher RA, Clardy J, Bargmann CI: **A Hub-and-Spoke Circuit Drives Pheromone Attraction and Social Behavior in C. elegans**. *Nature* 2009, **458**:1171-1175.
24. White JQ, Jorgensen EM: **Sensation in a single neuron pair represses male behavior in hermaphrodites**. *Neuron* 2012, **75**:593-600.
25. Park D, O'Doherty I, Somvanshi RK, Bethke A, Schroeder FC, Kumar U, Riddle DL: **Interaction of structure-specific and promiscuous G-protein–coupled receptors mediates small-molecule signaling in Caenorhabditis elegans**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:9917-9922.
26. McGrath PT, Xu Y, Ailion M, Garrison JL, Butcher RA, Bargmann CI: **Parallel evolution of domesticated Caenorhabditis species targets pheromone receptor genes**. *Nature* 2011, **477**:321-325.
27. Kim K, Sato K, Shibuya M, Zeiger DM, Butcher RA, Ragains JR, Clardy J, Touhara K, Sengupta P: **Two chemoreceptors mediate developmental effects of dauer pheromone in C. elegans**. *Science (New York, N.Y.)* 2009, **326**:994-998.
28. Zwaal RR, Mendel JE, Sternberg PW, Plasterk RHA: **Two Neuronal G Proteins Are Involved in Chemosensation of the Caenorhabditis Elegans Dauer-Inducing Pheromone**. *Genetics* 1997, **145**:715-727.
29. Larsen MJ, Lancheros ER, Williams T, Lowery DE, Geary TG, Kubiak TM: **Functional expression and characterization of the C. elegans G-protein-coupled FLP-2 Receptor (T19F4.1) in mammalian cells and yeast**. *International Journal for Parasitology, Drugs and Drug Resistance* 2013, **3**:1-7.
30. Tehseen M, Dumancic M, Briggs L, Wang J, Berna A, Anderson A, Trowell S: **Functional Coupling of a Nematode Chemoreceptor to the Yeast Pheromone Response Pathway**. *PLoS ONE* 2014, **9**:e111429.
31. Frézal L, Félix M-A: **C. elegans outside the Petri dish**. *eLife* 2015, **4**:e05849.
32. Palopoli MF, Peden C, Woo C, Akiha K, Ary M, Cruze L, Anderson JL, Phillips PC: **Natural and experimental evolution of sexual conflict within Caenorhabditis nematodes**. *BMC Evolutionary Biology* 2015, **15**:1-13.
33. Noble Luke M, Chang Audrey S, McNelis D, Kramer M, Yen M, Nicodemus Jasmine P, Riccardi David D, Ammerman P, Phillips M, Islam T, et al.: **Natural Variation in plep-1 Causes Male-Male Copulatory Behavior in C. elegans**. *Current Biology* 2015, **25**:2730-2737.

34. Leighton DHW, Choe A, Wu SY, Sternberg PW: **Communication between oocytes and somatic cells regulates volatile pheromone production in Caenorhabditis elegans**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:17905-17910.
35. Shinya R, Chen A, Sternberg PW: **Sex Attraction and Mating in Bursaphelenchus okinawaensis and B. xylophilus**. *Journal of Nematology* 2015, **47**:176-183.
36. Garcia LR, LeBoeuf B, Koo P: **Diversity in Mating Behavior of Hermaphroditic and Male–Female Caenorhabditis Nematodes**. *Genetics* 2007, **175**:1761-1771.
37. Kleemann GA, Basolo AL: **Facultative decrease in mating resistance in hermaphroditic Caenorhabditis elegans with self-sperm depletion**. *Animal Behaviour* 2007, **74**:1339-1347.
38. Aprison EZ, Ruvinsky I: **Sex Pheromones of C. elegans Males Prime the Female Reproductive System and Ameliorate the Effects of Heat Stress**. *PLoS Genet* 2015, **11**:e1005729.
39. McKnight K, Hoang HD, Prasain JK, Brown N, Vibbert J, Hollister KA, Moore R, Ragains JR, Reese J, Miller MA: **Neurosensory Perception of Environmental Cues Modulates Sperm Motility Critical for Fertilization**. *Science (New York, N.Y.)* 2014, **344**:754-757.
40. Duggal CL: **Initiation of Copulation and Its Effect On Oocyte Production and Life Span of Adult Female Panagrellus Redivivus**. *Nematologica* 1978, **24**:269-276.
41. Gems D, Riddle DL: **Longevity in Caenorhabditis elegans reduced by mating but not gamete production**. *Nature* 1996, **379**:723-725.
42. Diaz SA, Haydon DT, LindstrÖM JAN: **Sperm-limited fecundity and polyandry-induced mortality in female nematodes Caenorhabditis remanei**. *Biological Journal of the Linnean Society* 2010, **99**:362-369.
43. Carvalho S, Phillips PC, Teotónio H: **Hermaphrodite life history and the maintenance of partial selfing in experimental populations of Caenorhabditis elegans**. *BMC Evolutionary Biology* 2014, **14**:117-117.
44. Woodruff GC, Knauss CM, Maugel TK, Haag ES: **Mating Damages the Cuticle of C. elegans Hermaphrodites**. *PLoS ONE* 2014, **9**:e104456.
45. Shi C, Murphy CT: **Mating Induces Shrinking and Death in Caenorhabditis Mothers**. *Science* 2014, **343**:536-540.
46. Maures TJ, Booth LN, Benayoun BA, Izrayelit Y, Schroeder FC, Brunet A: **Males Shorten the Life Span of C. elegans Hermaphrodites via Secreted Compounds**. *Science* 2014, **343**:541-544.
47. Wu D, Tedesco PM, Phillips PC, Johnson TE: **Fertility/longevity trade offs under limiting-male conditions in mating populations of Caenorhabditis elegans**. *Experimental gerontology* 2012, **47**:759-763.

Chapter 2:

Communication between oocytes and somatic cells regulates volatile pheromone production in *C. elegans*

This chapter was originally published in the Proceedings of the National Academy of Sciences of the United States of America as:

Daniel H.W. Leighton, Andrea Choe, Shannon Y Wu, Paul W. Sternberg**: Communication between oocytes and somatic cells regulates volatile pheromone production in Caenorhabditis elegans**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111:17905-17910. doi: 10.1073/pnas.1420439111

http://www.pnas.org/content/111/50/17905.abstract

Abstract

Males of the androdioecious species *Caenorhabditis elegans* are more likely to attempt to mate with and successfully inseminate *C. elegans* hermaphrodites that do not concurrently bear sperm. Although a small number of genes have been implicated in this effect, the mechanism by which it arises remains unknown. In the context of the battle of the sexes, it is also unknown whether this effect is to the benefit of the male, the hermaphrodite, or both. We report that successful contact between mature sperm and oocyte in the *C. elegans* gonad, at the start of fertilization, causes the oocyte to release a signal that is transmitted to somatic cells in its mother, with the ultimate effect of reducing her attractiveness to males. Changes in hermaphrodite attractiveness are tied to the production of a volatile pheromone, the first such pheromone described in *C. elegans*.

Introduction

C. elegans' properties of self-fertilization and rapid generation, as well as the extensive library of *C. elegans* mutants, make it an excellent system for studying reproductive events. The generation time of *C. elegans* is under three days, and a single hermaphroditic worm can use its sperm to fertilize its own eggs, without any need for mating [1]. *C. elegans* and related nematodes possess a robust sperm sensation pathway that limits unfruitful oocyte maturation and ovulation [2]. Both self- and non-self-sperm secret protein ligands called Major Sperm Protein (MSP) that activate signal transduction pathways in both unfertilized oocytes (leading to activation of Mitogen Activated Protein kinase MPK-1) and the somatic gonad (involving transcription factor CEH-18). The result of this signaling is the release of oocytes from prophase I arrest and the ovulation of unarrested oocytes into the uterus [3,4].

Several behaviors of female and hermaphroditic nematodes have been demonstrated to correlate with either the presence of sperm or the recentness of mating. *C. elegans* hermaphrodites that have exhausted their supply of self-sperm are more likely to elicit a mating response from males of their species, and less likely to resist an attempted mating. Mutant *C. elegans* hermaphrodites that develop without self-sperm also elicit more mating attempts, and this increase in attractiveness vanishes after a successful mating [5,6]. In the gonochoristic species *Caenorhabditis brenneri* and *Caenorhabditis remanei*, males are attracted to a volatile pheromone produced only by females that have not recently mated [7]. The mechanisms that link these behaviors to sperm status remain unknown.

Pheromones have been shown to exist in dozens of nematode species [8-10], and positively identified in several, including *C. elegans* [11-14]. Although high performance liquid chromatography coupled with mass spectrometry has led to the identification of over 140 pheromones or pheromone-related metabolites in *C. elegans* [15], little is known about how production of such pheromones is regulated. Life stage and environmental conditions have been shown to affect pheromone output, but the detailed mechanisms remain elusive [16].

Results

Chasnov et al. [7] showed that females of *C. brenneri* and *C. remanei* produce an unknown volatile pheromone only if they have not recently been inseminated by conspecific males; *C. elegans* hermaphrodites produced no such pheromone. However, hermaphrodites of *C. elegans* reach adulthood already containing enough sperm to last nearly three days [17]. We hypothesized that a novel pheromone may be observed in *C. elegans* only when aging hermaphrodites exhaust their supply of self-sperm. We further set out to identify how sperm status regulates hermaphrodite behavior.

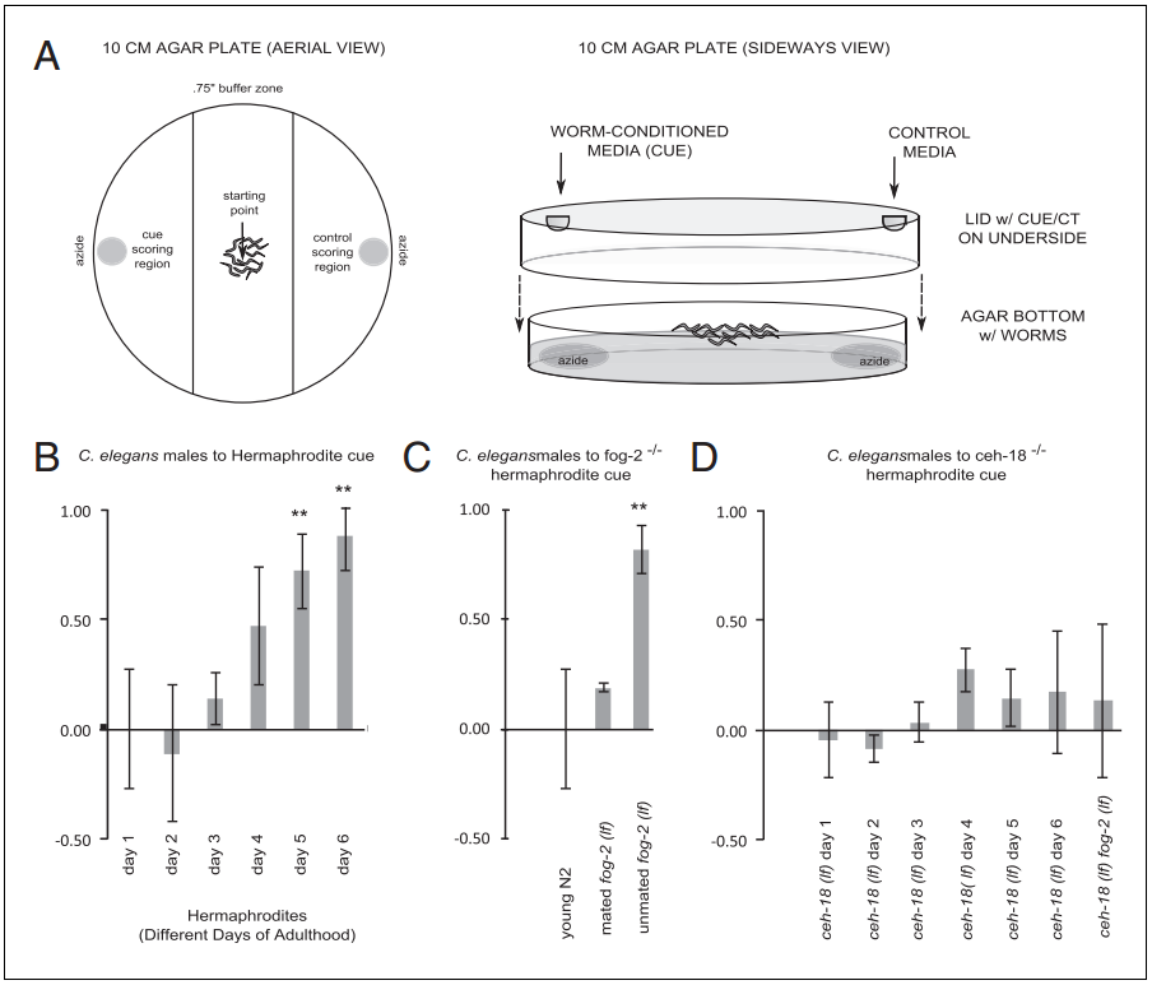

Figure 1: Volatile chemotaxis assays (y-axis values represent chemotaxis index). (A) Schematic of volatile chemotaxis assay. (B) Volatile chemotaxis of C. elegans males to adult hermaphrodites of varying age. Significance was determined by ANOVA: $F(5,17) = 9.460$, $p = 0.001$. Chemotaxis indices (CI) to WCM collected on days 5 and 6 are significantly greater ($p < 0.01$) than to the CI on day 1, as determined by Dunnett's post-test. (C) Volatile chemotaxis of C. elegans males to mated fog-2 mutants versus unmated mutants and wild-type hermaphrodites. Attraction to mated fog-2 mutants is significantly less than attraction to unmated mutants ($p < 0.0005$), but statistically indistinguishable from attraction to wild-type hermaphrodites ($p > 0.1$). Significance was determined by the one-tailed, homoscedastic student's t-test. (D) Chemotaxis of young adult males to ceh-18 mutant hermaphrodites in the 1st-6th day of adulthood and pseudofemales in the first day of adulthood. No significant differences were found.

To test our hypothesis, we generated a synchronized population of larval hermaphrodites and allowed them to mature into adults. On the day that adulthood was reached and the next five days, a fraction of aging worms was incubated in a buffered medium to collect pheromone. Upon completion of the collection, the worms were removed, yielding worm conditioned media (WCM). To test for the presence of volatile pheromone, *C. elegans* males were placed in a

chemotaxis assay in which the WCM was not permitted to come into direct or indirect physical contact with the males (Fig. 1A). Any attraction to the WCM would necessarily result from sensation of volatile compounds. Our results show that males are attracted to a volatile compound produced by hermaphrodites, but only on the 5^{th}-6^{th} days of adulthood (Fig. 1B). Prior research shows that sperm exhaustion occurs on the 3^{rd} day [17], consistent with our own observations. Control experiments confirmed that the volatile pheromone is neither attractive to hermaphrodites nor produced by males (Fig. 3A,B).

To confirm that our findings resulted from loss of sperm rather than any other feature of aging, we collected WCM from hermaphrodites bearing a mutation that eliminates hermaphrodite sperm production (*fog-2*) [18]. We also collected WCM from feminized hermaphrodites that had been mated to fertile males. Non-self-sperm are also capable of triggering suppression of pheromone production, causing the mutant hermaphrodites to attract only 19% of males versus 82% attracted by unmated mutants (Fig. 1C). We also performed interspecies chemotaxis assays with *C. remanei* to see whether the *C. elegans* pheromone would function cross-species. We found that the two species' volatiles are interchangeable – males of each species are attracted to females of each species (Fig. 3C).

These initial experiments indicate that a volatile pheromone is produced by hermaphrodites and attracts males (Fig. 1B). This pheromone is produced only after a hermaphrodite's self-sperm supply has been exhausted. Since *fog-2* mutants, which lack self-sperm, produce the pheromone on the first day of adulthood, we can infer that pheromone production is linked to sperm status rather than age (Fig. 1C). The finding that mated *fog-2* mutants do not attract males suggests that initiation of pheromone production is reversible.

This correlation between spermlessness and pheromone production in *C. elegans* hermaphrodites is inconsistent with the findings of Chasnov et al. [7], who also prepared WCM from aged hermaphrodites and spermless mutants. However, our WCM preparation protocol calls for twenty times as many worms per unit volume, and collection occurs for four times as long, potentially yielding a far more concentrated product. Our chemotaxis assays also differ markedly.

To test the similarity of our volatile cue to the unknown mating cue suggested by Morsci et al.[6], which also correlates with age, we collected WCM from hermaphrodites and feminized worms mutant for *ceh-18*, a transcription factor gene found to be essential for production of this cue [6]. We found no detectable volatile attraction of males to *ceh-18* mutant hermaphrodites or females of any age (Fig. 1D). We therefore conclude that our pheromone's production is dependent on CEH-18. However, since *ceh-18* is a poorly understood gene that is expressed in multiple cell types, we cannot draw more detailed conclusions from this result.

To study the regulation of the volatile pheromone, we tested a series of mutant strains defective in various aspects of reproduction. We chose mutations that cause germline development and reproduction to cease at a defined point so that we could determine which specific aspect of the reproductive process regulates production of the volatile cue.

Our results show a striking anti-correlation between sperm-egg contact and volatile pheromone production (Fig. 2). How specific reproductive phenotypes correlate with pheromone production reveals details about the regulation of this pheromone (Table 1). Mutations that induced inappropriate pheromone production include those in the genes *glp-1*, *fem-2*, *fog-2*, *spe-8*,and *egg-3* as well as the *oma-1*; *oma-2* double mutant. Tested mutations that did not induce

pheromone production include those in genes *spe-42*, *mbk-2*, *chs-1*, *mei-1* and *pos-1* as well as the *egg-4*; *egg-5* double mutant.

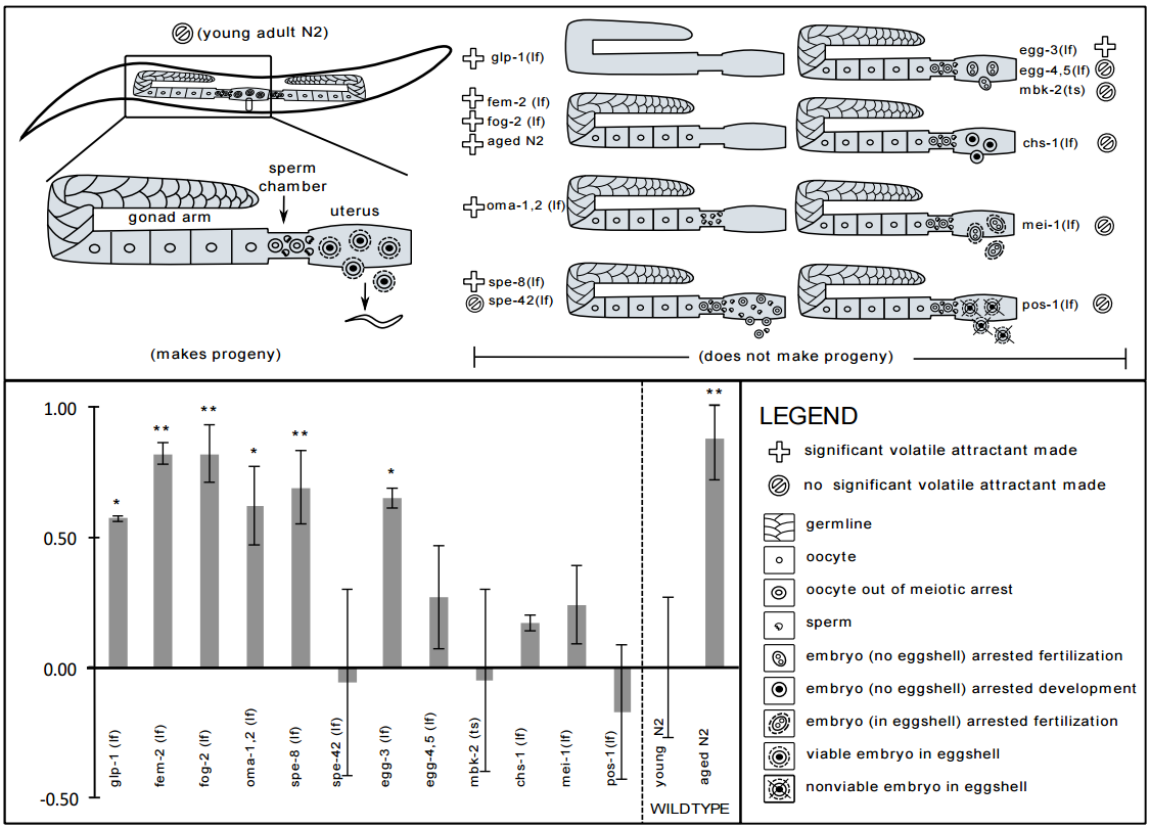

Figure 2: Relationship of germline status to pheromone output. Volatile chemotaxis of C. elegans males to hermaphrodites of various genotypes, accompanied by a diagram of germline status in each of the strains used in the mutant survey. Significance of the data set was determined by ANOVA: $F(13,50) = 9.319$, $p < 0.0001$, with significant differences from wild type determined by Dunnett's post-test ($p < 0.05$ is indicated by *; $p < 0.01$ is indicated by **). In general, fertilization and volatile pheromone production are anti-correlated. WCM for all mutant strains except for aged N2 hermaphrodites was prepared from young adults. Chemotaxis assays for spe-42 and egg-4; egg-5 double were performed six times instead of three, due to high variance.

The results of this mutant survey (Fig. 2) reveal that the phenotype of the worm may be predicted by the developmental time at which the mutated gene acts. *C. elegans* germline development begins with two unsexed, primordial germ cells (PGC) [19]. These cells divide mitotically to produce cells that will eventually develop into spermatocytes during the fourth larval stage and oocytes during adulthood. Oocytes arrest during prophase I, and do not continue meiosis at high rates until activated by MSP secreted by spermatozoa. The oocytes

reinitiate meiosis at the same time that they are ovulated in preparation for sperm entry. Activated oocytes re-arrest if they are not fertilized by spermatozoa. Ovulated oocytes that are not fertilized are laid by the hermaphrodite as if they were eggs [2].

We first examined a mutant with complete failure of germline proliferation, leading to adults with virtually no gametes of either sex (*glp-1*) [[20]. The production of pheromone by *glp-1* mutants, in the absence of any appreciable germline, implies that the pheromone is produced by the soma; the default state of the soma is to produce, and a normal germline suppresses volatile pheromone production. We then tested a mutation that feminizes the germline, leading to adults with normal oocytes but no sperm (*fem-2*) [21]. As with the previous experiment using *fog-2* mutants, *fem-2* mutant hermaphrodites produce pheromone in young adulthood, corroborating the finding that absence of sperm is sufficient to trigger pheromone production, and aging is unnecessary.

In addition to looking at the mere presence of sperm, we examined three strains in which both sperm and oocytes are present but their interactions are impaired. In the first strain (*oma-1*; *oma-2* double mutant), ovulation never occurs, and so the two types of gametes never meet [22]. The production of pheromone by the *oma-1*; *oma-2* double mutant indicates that the presence of normal sperm is insufficient to suppress pheromone production. In the second strain (defective in *spe-8*), sperm are immobile and cannot fertilize oocytes, although they are fully capable of stimulating meiotic activation, ovulation, and egg-laying [23]. Since this strain also produces pheromone, we know that activation of the canonical sperm sensation pathway is insufficient to suppress pheromone production. Finally, we tested a strain (defective in *spe-42*) containing superficially normal sperm and oocytes, although fertilization does not continue past sperm-egg contact [24]. This strain failed to produce pheromone in young adulthood, making sperm-egg fusion the earliest failure in reproduction that still permits pheromone suppression.

We also examined the complex of genes known to regulate oocyte activities during fertilization. Strains bearing these mutations produce and lay eggs that failed to undergo pronuclear fusion and/or have no shells. The strains studied are *egg-3*, simultaneous mutation of *egg-4* and *egg-5*, *mei-1*, *mbk-2*, and *chs-1* [25-29]. We further looked at one strain that displays completely normal fertilization, but whose embryos fail soon thereafter (*pos-1*) [30].

Upon sperm-egg fusion in a wild-type worm, the presence of the sperm is signaled by a complex whose components are encoded by *egg-3*, *egg-4*, and *egg-5*. This complex triggers the completion of meiosis and is also required for proper assembly of the egg shell [2]. Our data demonstrate that EGG-3 is required for pheromone suppression, but its two binding partners as well as its known substrates, MBK-2 and CHS-1, are dispensable for pheromone suppression. The genes *mei-1* and *pos-1*, required for meiosis and embryonic cell determination, respectively, are also unnecessary for suppression of volatile pheromone production.

To test whether EGG-3 has an undescribed role in sperm, we also collected WCM from *egg-3(-/-)* hermaphrodites that had been mated to fertile males, so that they would contain a supply of *egg-3(+/+)* sperm. These animals continue to produce volatile pheromone, showing that *egg-3(+/+)* sperm are insufficient to rescue the attraction phenotype of *egg-3(-/-)* hermaphrodites (Fig. S1).

To determine whether the vulva is required for volatile pheromone release, we produced feminized animals that lack a vulva (a *let-23(sy1)*; *lin-18(e620)* double mutant treated with *fem-1* RNAi). This strain continues to produce and release volatile pheromone (Fig. 3D).

In an effort to identify genes involved in sperm-oocyte communication, we assayed genes that code for transmembrane proteins required for fertilization: *spe-9*, *spe-41*, and *egg-1*. In all three cases, no pheromone production was detected (Fig. 3E).

Discussion

Our data indicate that simultaneous presence of mature sperm and activated oocytes in the spermatheca (the portion of the gonad in which sperm are stored and fertilization takes place), which occurs just prior to fertilization, is necessary for suppression of the volatile pheromone. Membrane fusion is not required.

Table 1. Results and implications of Fig. 2

Strain	Sperm status	Oocyte status	Volatile pheromone	Implications
glp-1	Very few, normal	None	Yes	Pheromone is produced by the soma and suppressed by the germ line.
fem-2	None	Normal	Yes	Sperm depletion eliminates suppression without aging.
fog-2	None	Normal	Yes	Same as *fem-2*.
Aged N2	None	Normal	Yes	Aging of WT worms eliminates suppression.
oma-1,2	Normal	Cannot be ovulated	Yes	Sperm presence is insufficient.
spe-8	Immobile	Normal, ovulated	Yes	Oocyte activation/ovulation is insufficient.
spe-42	Cannot fuse	Normal, ovulated	No	Sperm–oocyte fusion is unnecessary.
egg-3	Normal	Oocyte cannot react to sperm	Yes	EGG-3 is necessary.
egg-4,5	Normal	Oocyte cannot react to sperm	No	Known EGG-3–binding partners are unnecessary.
mbk-2	Normal	No eggshell, meiosis incomplete	No	Known downstream effectors of EGG-3 are unnecessary.
chs-1	Normal	No eggshell	No	Eggshell deposition is unnecessary.
mei-1	Normal	Meiosis incomplete	No	Completion of meiosis is unnecessary.
pos-1	Normal	Resulting embryo dies	No	Embryonic viability is unnecessary.
Young N2	Normal	Normal	No	Young WT worms suppress pheromone output.

The terms "insufficient", "necessary," and "unnecessary" are always in reference to the suppression of pheromone production. Overall, we conclude that the pheromone is produced in the soma and suppressed by a signal originating in the germ line. EGG-3 and sperm–oocyte contact are necessary for suppression. Eggshell deposition, complete meiosis, known EGG-3 effectors, known EGG-3–binding partners, and embryonic viability are unnecessary for suppression. Sperm presence, oocyte activation, ovulation, and egg/oocyte-laying are insufficient for suppression.

The signal to suppress volatile pheromone production originates from the activated oocyte in an EGG-3-dependent manner, and is transmitted to the maternal soma. The mere presence of sperm, activation and ovulation of oocytes, egg-laying, and sperm-oocyte contact is insufficient for pheromone suppression. Complete meiosis, embryonic viability, and the known effectors and binding partners of EGG-3 are unnecessary for pheromone suppression (Table 1).

Our data cannot distinguish between a scenario in which mature sperm and oocyte simultaneously signal to somatic cells, and one in which mature sperm and oocyte communicate with one another before just a single germ cell-type signals somatic tissue. In an effort to investigate the communication scheme, we tested a series of mutants that are defective for transmembrane proteins involved in fertilization, but are not known to significantly impact

oocyte or sperm development. A failure of any of these strains to suppress pheromone would suggest the mutant gene is in the suppression pathway, and may have led to an understanding of how sperm status is communicated. The mutants chosen were *spe-9*, *spe-41*, and *egg-1*, which reduce successful fertilization to very low rates [31-33]. None of these mutations prevented suppression of volatile pheromone production (Fig. 3E). We hypothesize that if sperm surface proteins are necessary for suppression, that this is accomplished through surface ligands that have not been implicated in fertilization. Since the mutations for *spe-9* and *egg-1* are temperature sensitive rather than nulls, as is the case for *spe-41*, they may retain limited function even at the non-permissive temperature. A low rate of successful sperm-oocyte contact might have masked the requirement for either *spe-9* or *egg-1* in pheromone suppression. Our data does not provide any suggestions for the nature of the oocyte-soma or sperm-soma signal.

These results are especially confusing in light of the dependence of pheromone suppression on the action of EGG-3. If the sperm are unable to fuse to the oocyte, EGG-3's known functions should fail to be carried out. Further, EGG-3's known functions are dependent on the presence of either EGG-4 or EGG-5, as well as downstream proteins MEI-1, CHS-1, and MBK-2. That none of the proteins tested in this pathway influence pheromone production, whether they reside in the sperm or the egg, suggests a novel and independent function for EGG-3. This issue is even further confused by the fact that localization of EGG-3 to the oocyte cortex is dependent on the presence of CHS-1[25]. If EGG-3 carries out an undescribed function in pheromone regulation, it is a function that does not require its typical localization. This all unfortunately leaves us with no clear hypothesis for how EGG-3 regulates pheromone production.

The similarity of our results to those of Morsci *et alia* on the mating preference cue [6] lead us to further speculate that the volatile pheromone and the mating-preference cue are regulated by the same process, and may be the same.

We naturally question what drove the evolution of oocyte-to-soma communication, which had previously only been reported in mammals, where communication between somatic cells and oocytes or embryos is critical to embryonic survival. In addition to influencing events that occur in both uterine and embryonic tissue shortly after fertilization, oocyte-uterine signaling plays a large role in modifying the nutrition and development of the fetus [34,35]. In nematodes, however, the mother plays no role in the embryo's nutrition and development after fertilization; therefore, such communication would serve no purpose to the developing embryo. Our findings suggest a novel utility for oocyte-uterine signaling – one which may be related to the exclusive benefit of the mother.

A growing body of research has demonstrated that the presence of males and the act of mating may be hazardous to the health of females and hermaphrodites in the genus *Caenorhabditis*. Mating between hermaphrodites and males, and even mere exposure to male secretions, has been shown to shorten the life-span of *C. elegans* hermaphrodites [36,37]. Male sperm and seminal fluid also trigger physiological changes in mated hermaphrodites [38]. It has also been shown that the act of mating is followed by the appearance of cuticular damage around the hermaphrodite vulva [39]. Unfortunately it is unclear whether any of these physiological and lifespan alterations are detrimental to hermaphrodite fecundity or the fitness of their offspring. It is also unclear whether these changes provide any benefit to the male mating partner or its offspring. In other species of *Caenorhabditis*, however, a clear danger of mating has been demonstrated. Males of the species *C. nigoni* have been shown to sterilize and kill hermaphrodites and females of other species through the action of their sperm [40].

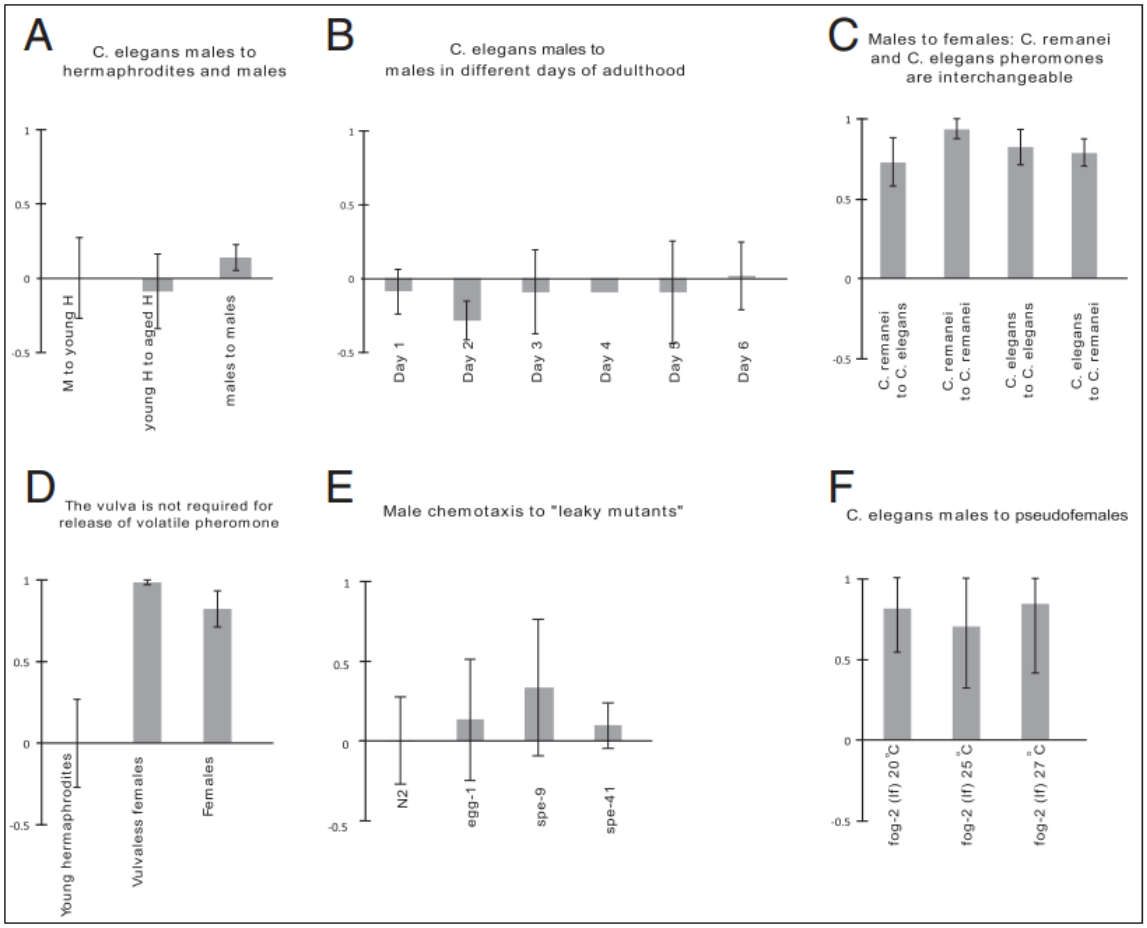

Figure 3: Controls and other minor experiments. y-axis values represent chemotaxis index**.** (*A*) Chemotaxis of young adult males to young adult hermaphrodites, young hermaphrodites to aged hermaphrodites, and young adult males to young adult males. No significant differences were found in any pairwise comparison. (*B*) Chemotaxis of young adult males to males in the 1st-6th day of adulthood. No significant differences were found. (Error bar for day 4 is not shown as the standard deviation is very small: 0.016.). (*C*) Chemotaxis of young adult males of *C. remanei* and *C. elegans* to females and aged hermaphrodites of *C. remanei* and *C. elegans*. No significant differences were found. (*D*) Attraction of young adult males to vulvaless pseudofemales is not significantly less than attraction to otherwise normal pseudofemales. (*E*) Attraction of young adult males to incompletely sterile mutants defective for fertilization. No significant differences were found when compared with attraction to young adult hermaphrodites. (*F*) Attraction of young adult males to pseudofemales raised at varying temperatures. No significant differences were found.

We hypothesize that due to the potential harms of mating, it benefits the mother to recognize when successful fertilization has occurred, therefore shutting off further production of volatile pheromone. While we have been presenting a scenario in which mating benefits males and harms hermaphrodites (if they already possess sperm), it may also be the case that the male

benefits from this arrangement by minimizing the possibility of sperm competition. There may also be reasons to shut off pheromone production unrelated to mating control, such as limiting the number of worms who may encroach on the hermaphrodite's food source.

It is interesting to note that previous work shows that gravid hermaphrodites produce non-volatile mate-attracting pheromones [41]. We cannot fully explain why, if production of mating pheromones is disadvantageous to the hermaphrodite, they continue to produce non-volatile attractants. It is possible that the loss of production of volatile attractants serves to reduce the range of attraction, rather than abolish it completely. However, it is difficult to speculate on the significance of the volatility of *C. elegans* pheromones as it remains unclear in what environment the species evolved [42]. It is also possible that the non-volatile attractants are indispensable for reasons other than mate-attraction; indeed, some of these attractants induce dauer formation[41]. It is further possible that output of non-volatile attractants is reduced as a result of sperm depletion; the appropriate experiments to show this have not been performed. However, we have provided the most detailed characterization yet of the regulation of pheromone output in a nematode, and we hope that this will lead to greater understanding of how nematodes communicate through chemical signals.

Materials and Methods

Strains. Provided by the *Caenorhabditis* Genomics Center (complete genotypes available at http://www.cgc.cbs.umn.edu/): N2 (*C. elegans* wild type), EM464 (*C. remanei* wild type), BA671 (*spe-9*), PS4330 (*spe-41*), AD186 (*egg-1*), GR1034 (*ceh-18*), BA785 (*spe-8*), JK574 (*fog-2*), DG1604 (*fog-2*; *ceh-18*), VC2876 (*egg-3*), AD266 (*egg-4*; *egg-5*), JJ462 (*pos-1*), TX183 (*oma-1*; *oma-2*), SL1138 (*spe-42*), RB1189 (*chs-1*), TH48 (*mbk-2*), VC1530 (*mei-1*), CB1490 (*him-5*). Provided by H.

Robert Horvitz: MT11436 (*fem-2(e2105ts,mat) III / sC1 III [s2023 dpy-1(s2170) III]*). Provided by Paul Minor (Sternberg laboratory): PS6345 (*let-23(sy1)*; *lin-18(e620)*).

Strain maintenance. Worms were raised on NG-agar plates that had been seeded with *E. coli* OP50. The males used for chemotaxis assays are collected from strain CB1490. For the purpose of WCM collection, TH48, AD186, and BA671 were grown at the non-permissive temperature of 25°C and 15°C otherwise. All other strains were grown at room temperature. Control experiments confirm that raising worms at high temperature does not interfere with pheromone production (Fig. 3f).

Synchronization. Males for chemotaxis assays and hermaphrodites for WCM preparation were synchronized by time of hatching prior to other procedures. Synchronization was carried out over two generations. In the first generation, a plate of worms was rinsed with M9 buffer, removing larvae and adults but leaving behind eggs. The roughly synchronized larvae resulting from these eggs were permitted to grow to adulthood and lay eggs of their own. As the second generation of larvae began to hatch from these eggs, all worms were removed from the plate in M9. The adults and arbitrarily synchronized larvae were separated from one another by relative buoyancy, after which the larvae were replated for later use.

Worm conditioned media. WCM was prepared using synchronized young-adult hermaphrodites and pseudofemales. In the case of strains that segregate genotypes, the desired hermaphrodites were separated from the origin strain by picking. For strains that did not produce males, worms were picked on the day of collection. For strains that also produced males, worms were picked the day before collection as L4s. On the day of collection, worms were rinsed from their plate in M9 buffer and permitted to settle to the bottom of a microcentrifuge tube. Upon settling, the supernatant was removed and the worms were resuspended (or rinsed) in fresh M9. This rinsing

step was performed a total of four times to remove bacteria from the worms. When the rinsing was complete, the worms were placed in the inverted cap of a microcentrifuge tube, one worm per microliter, 75 worms per cap. The cap was sealed and permitted to sit at room temperature for 24 hours, after which the worms and any eggs or larvae deposited in the WCM were removed by centrifugation. WCM was stored at -20°C.

Chemotaxis assays. Chemotaxis assays were performed with synchronized males unless otherwise noted. Males were separated from hermaphrodites by picking during the young-adult stage, and permitted to feed for another 24 hours. This segregation procedure is meant to reduce the influence of recent mating on male chemotaxis behavior. The males were then rinsed from their plate with M9 buffer and transferred to a microcentrifuge tube. Rinsing was performed in the same manner as with WCM preparation, but only twice with M9 to remove bacteria and then twice with ddH_2O to remove salts. The chemotaxis assay was performed in a 10cm Petri dish prepared with chemotaxis agar (1.5% agar, 75 mM NH_4Cl, 10 mM MOPS, pH 7.2 with NH_4OH, and 0.25% Tween 20). During the final rinse of the males, one microliter of sodium azide was dropped on each far side of the plate (referred to as east and west), a diameter apart. (Sodium azide functions as a rapidly lethal neurotoxin when encountered by *C. elegans*, allowing us to record which position the male was most attracted to, rather than which position he is more likely to linger at if he is sampling without initial bias. Since the azide is added to both sides of the plate, and has not had time to diffuse through the agar when the assays begin, we do not believe it has any effect on experimental outcomes.) Once the final rinse was complete, 100 males were pipetted onto the center of the plate, and gently spread north and south to speed up the absorption of the excess water into the agar. As the water began to absorb, ten microliters of WCM was pipetted to the inside lid of the Petri dish above one spot of azide, and ten microliters of M9 was pipetted to the other. While thawing and pipetting the WCM, we

were careful to pipette from the top of the media and not to disturb or mix the media. The plate was then placed in a small box to eliminate the influence of light, and onto a vibration resistant platform to reduce the influence of vibrations. The assay was run until ≥95% of the males were dead, as determined by periodic checking. This generally took between 15 minutes and four hours. At the conclusion of the assay, the males were tallied on the east and west sides of the plate, while ignoring all worms within a 0.75 inch buffer zone along the north-south axis. Chemotaxis index is computed as **(cue – control)/(cue+control)**. All chemotaxis assays were repeated three times unless otherwise noted.

Hermaphrodite aging. A population of 15,000 synchronized N2 larvae was permitted to reach young adulthood, split across several plates. On the day that young adulthood was reached, a portion of the worms were put into WCM collection, with the rest being replated by rinsing onto fresh food. On each subsequent day until the sixth day, this procedure was repeated but with an additional step of separating the adults from eggs and larvae by relative buoyancy. On the sixth day, all remaining adults were put into WCM collection.

Mating assay. The testing of mated versus unmated *fog-2(lf)* animals was performed with strain JK574. For the unmated population, 150 L4 pseudofemales were segregated to a separate plate and permitted to grow for another 24 hours, at which point the resulting adults were put into WCM collection. For the mated population, 150 L4 pseudofemales and an equal number of otherwise isogenic males were permitted to grow on the same plate for 24 hours. At the conclusion of the mating period, males were discarded and the pseudofemales were put into WCM collection. The testing of mated versus unmated *egg-3(lf)* animals was performed in otherwise identical fashion.

Acknowledgements. We thank Asher Cutter and Eric Haag for communicating unpublished results; Ashley Hebard for providing assistance on some experiments; Mihoko Kato and Hillel Schwartz for helpful discussions; and H. Robert Horvitz, Paul Minor, and the Caenorhabditis Genetics Center for sharing worm strains. D.L. was supported by a National Institutes of Health United States Public Health Service Training Grant (T32GM07616). Research supported by the Howard Hughes Medical Institute, with which P.W.S. is an investigator.

Author's Contributions. A.C. developed the volatile assay. D. L. and A.C. designed the experiments. D.L. and S.W. performed the assays. D.L., A.C., and P.S. wrote the paper.

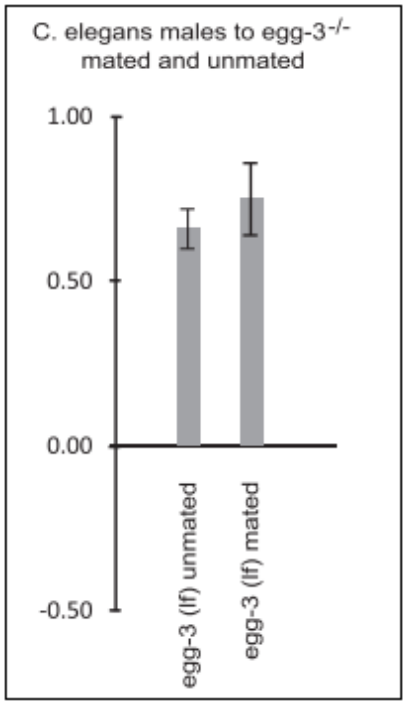

Figure: S1. Effects of mating on pheromone output of *egg-3* mutant hermaphrodites. y-axis represents chemotaxis index. Chemotaxis of young adult males to young adult hermaphrodites mutant for *egg-3* is unaffected by whether the hermaphrodites have been mated. Attraction showed no significant difference between mated and unmated samples.

References

1. Brenner S: **THE GENETICS OF CAENORHABDITIS ELEGANS**. *Genetics* 1974, **77**:71-94.

2. Singaravelu G, Singson A: **Chapter six - New Insights into the Mechanism of Fertilization in Nematodes**. In *International Review of Cell and Molecular Biology*. Edited by Kwang WJ: Academic Press; 2011:211-238. vol Volume 289.]
3. Miller MA, Nguyen VQ, Lee M-H, Kosinski M, Schedl T, Caprioli RM, Greenstein D: **A Sperm Cytoskeletal Protein That Signals Oocyte Meiotic Maturation and Ovulation**. *Science* 2001, **291**:2144-2147.
4. Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D: **An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in Caenorhabditis elegans**. *Genes & Development* 2003, **17**:187-200.
5. Kleemann GA, Basolo AL: **Facultative decrease in mating resistance in hermaphroditic Caenorhabditis elegans with self-sperm depletion**. *Animal Behaviour* 2007, **74**:1339-1347.
6. Morsci NS, Haas LA, Barr MM: **Sperm Status Regulates Sexual Attraction in Caenorhabditis elegans**. *Genetics* 2011, **189**:1341-1346.
7. Chasnov JR, So WK, Chan CM, Chow KL: **The species, sex, and stage specificity of a Caenorhabditis sex pheromone**. *Proceedings of the National Academy of Sciences* 2007, **104**:6730-6735.
8. MacKinnon BM: **Sex Attractants in Nematodes**. *Parasitology Today* 1987, **3**:156-158.
9. Green CD: **Nematode sex attractants**. *Helminthological Abstracts, Series B* 1980, **49**:81-93.
10. Choe A, von Reuss Stephan H, Kogan D, Gasser Robin B, Platzer Edward G, Schroeder Frank C, Sternberg Paul W: **Ascaroside Signaling Is Widely Conserved among Nematodes**. *Current biology : CB* 2012, **22**:772-780.
11. Jaffe H, Huettel RN, Demilo AB, Hayes DK, Rebois RV: **Isolation and identification of a compound from soybean cyst nematode *Heterodera glycines*, with sex pheromone activity**. *Journal of Chemical Ecology* 1989, **15**:2031-2043.
12. Choe A, Chuman T, von Reuss SH, Dossey AT, Yim JJ, Ajredini R, Kolawa AA, Kaplan F, Alborn HT, Teal PEA, et al.: **Sex-specific mating pheromones in the nematode Panagrellus redivivus**. *Proceedings of the National Academy of Sciences* 2012, **109**:20949-20954.
13. Noguez JH, Conner ES, Zhou Y, Ciche TA, Ragains JR, Butcher RA: **A Novel Ascaroside Controls the Parasitic Life Cycle of the Entomopathogenic Nematode Heterorhabditis bacteriophora**. *ACS Chemical Biology* 2012.
14. Jeong P-Y, Jung M, Yim Y-H, Kim H, Park M, Hong E, Lee W, Kim YH, Kim K, Paik Y-K: **Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone**. *Nature* 2005, **433**:541-545.
15. von Reuss SH, Bose N, Srinivasan J, Yim JJ, Judkins JC, Sternberg PW, Schroeder FC: **Comparative Metabolomics Reveals Biogenesis of Ascarosides, a Modular Library of Small-Molecule Signals in C. elegans**. *Journal of the American Chemical Society* 2012, **134**:1817-1824.
16. Kaplan F, Srinivasan J, Mahanti P, Ajredini R, Durak O, Nimalendran R, Sternberg PW, Teal PEA, Schroeder FC, Edison AS, et al.: **Ascaroside Expression in Caenorhabditis elegans Is Strongly Dependent on Diet and Developmental Stage**. *PLoS ONE* 2011, **6**:e17804.
17. Byerly L, Cassada RC, Russell RL: **The life cycle of the nematode Caenorhabditis elegans: I. Wild-type growth and reproduction**. *Developmental Biology* 1976, **51**:23-33.
18. Schedl T, Kimble J: **fog-2, a Germ-Line-Specific Sex Determination Gene Required for Hermaphrodite Spermatogenesis in Caenorhabditis elegans**. *Genetics* 1988, **119**:43-61.
19. Hubbard EJA, Greenstein D: **Introduction to the Germline**. In *WormBook*. Edited by: Edited by: The *C. elegans* Research Community. WormBook; 2005.

20. Austin J, Kimble J: **glp-1 Is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans**. *Cell* 1987, **51**:589-599.
21. Pilgrim D, McGregor A, Jäckle P, Johnson T, Hansen D: **The C. elegans sex-determining gene fem-2 encodes a putative protein phosphatase**. *Molecular Biology of the Cell* 1995, **6**:1159-1171.
22. Detwiler MR, Reuben M, Li X, Rogers E, Lin R: **Two Zinc Finger Proteins, OMA-1 and OMA-2, Are Redundantly Required for Oocyte Maturation in C. elegans**. *Developmental Cell* 2001, **1**:187-199.
23. L'Hernault SW, Shakes DC, Ward S: **Developmental Genetics of Chromosome I Spermatogenesis-Defective Mutants in the Nematode Caenorhabditis elegans**. *Genetics* 1988, **120**:435-452.
24. Kroft TL, Gleason EJ, L'Hernault SW: **The spe-42 gene is required for sperm–egg interactions during C. elegans fertilization and encodes a sperm-specific transmembrane protein**. *Developmental Biology* 2005, **286**:169-181.
25. Maruyama R, Velarde NV, Klancer R, Gordon S, Kadandale P, Parry JM, Hang JS, Rubin J, Stewart-Michaelis A, Schweinsberg P, et al.: **EGG-3 Regulates Cell-Surface and Cortex Rearrangements during Egg Activation in Caenorhabditis elegans**. *Current biology : CB* 2007, **17**:1555-1560.
26. Parry JM, Velarde NV, Lefkovith AJ, Zegarek MH, Hang JS, Ohm J, Klancer R, Maruyama R, Druzhinina MK, Grant BD, et al.: **EGG-4 and EGG-5 Link Events of the Oocyte-to-Embryo Transition with Meiotic Progression in C. elegans**. *Current biology : CB* 2009, **19**:1752-1757.
27. Zhang Y, Foster JM, Nelson LS, Ma D, Carlow CKS: **The chitin synthase genes chs-1 and chs-2 are essential for C. elegans development and responsible for chitin deposition in the eggshell and pharynx, respectively**. *Developmental Biology* 2005, **285**:330-339.
28. Clark-Maguire S, Mains PE: **mei-1, a gene required for meiotic spindle formation in Caenorhabditis elegans, is a member of a family of ATPases**. *Genetics* 1994, **136**:533-546.
29. Pellettieri J, Reinke V, Kim SK, Seydoux G: **Coordinate Activation of Maternal Protein Degradation during the Egg-to-Embryo Transition in C. elegans**. *Developmental Cell* 2003, **5**:451-462.
30. Tabara H, Hill RJ, Mello CC, Priess JR, Kohara Y: **pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans**. *Development* 1999, **126**:1-11.
31. Xu XZS, Sternberg PW: **A C. elegans Sperm TRP Protein Required for Sperm-Egg Interactions during Fertilization**. *Cell* 2003, **114**:285-297.
32. Singson A, Mercer KB, L'Hernault SW: **The C. elegans spe-9 Gene Encodes a Sperm Transmembrane Protein that Contains EGF-like Repeats and Is Required for Fertilization**. *Cell* 1998, **93**:71-79.
33. Kadandale P, Stewart-Michaelis A, Gordon S, Rubin J, Klancer R, Schweinsberg P, Grant BD, Singson A: **The Egg Surface LDL Receptor Repeat-Containing Proteins EGG-1 and EGG-2 Are Required for Fertilization in Caenorhabditis elegans**. *Current Biology* 2005, **15**:2222-2229.
34. van Mourik MSM, Macklon NS, Heijnen CJ: **Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment**. *Journal of Leukocyte Biology* 2009, **85**:4-19.
35. Murphy VE, Smith R, Giles WB, Clifton VL: **Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus**. *Endocrine Reviews* 2006, **27**:141-169.

36. Gems D, Riddle DL: **Longevity in Caenorhabditis elegans reduced by mating but not gamete production**. *Nature* 1996, **379**:723-725.
37. Maures TJ, Booth LN, Benayoun BA, Izrayelit Y, Schroeder FC, Brunet A: **Males Shorten the Life Span of C. elegans Hermaphrodites via Secreted Compounds**. *Science* 2014, **343**:541-544.
38. Shi C, Murphy CT: **Mating Induces Shrinking and Death in Caenorhabditis Mothers**. *Science* 2014, **343**:536-540.
39. Woodruff GC, Knauss CM, Maugel TK, Haag ES: **Mating Damages the Cuticle of C. elegans Hermaphrodites**. *PLoS ONE* 2014, **9**:e104456.
40. Ting JJ, Woodruff GC, Leung G, Shin N-R, Cutter AD, Haag ES: **Intense Sperm-Mediated Sexual Conflict Promotes Reproductive Isolation in <italic>Caenorhabditis</italic> Nematodes**. *PLoS Biol* 2014, **12**:e1001915.
41. Srinivasan J, Kaplan F, Ajredini R, Zachariah C, Alborn HT, Teal PEA, Malik RU, Edison AS, Sternberg PW, Schroeder FC: **A blend of small molecules regulates both mating and development in *Caenorhabditis elegans***. *Nature* 2008, **454**:1115-1118.
42. Caswell-Chen EP, Chen J, Lewis EE, Douhan GW, Nadler SA, Carey JR: **Revising the Standard Wisdom of C. elegans Natural History: Ecology of Longevity**. *Sci. Aging Knowl. Environ.* 2005, **2005**:pe30-.

Chapter 3:

Attempts to identify pheromone regulatory genes through random mutagenesis and RNAseq

Introduction

With the existence of a novel pheromone demonstrated, it is desirable to uncover the mechanisms behind its regulation. However, the discoveries detailed in Chapter 2 of this thesis leave very little to go on. Although it is appears probable that the default state of the soma is to produce pheromone, and the repression pathway involves *egg-3*, we do not know enough to place this novel pheromone on a known pathway. Experiments involving the genes *mbk-2*, *egg-4*, *egg-5*, *mei-1*, and *chs-1* demonstrate that the known *egg-3* pathways have no apparent involvement in pheromone regulation.

In a broader context, there are no known biochemical pathways comparable to what our data suggests – a response of somatic cells to fertilization of an egg – in any organism other than mammals. A thorough review of the literature, as well as correspondence with experts in the field, has yielded no reports of such a pathway, or even hints of one: insects are not known to behave differently after mating according to the ability of sperm to fertilize an egg; birds and lizards care for unfertilized eggs the same as fertilized ones (at least until the egg begins to rot).

We therefore chose to approach this problem through two methods that encompass the entire genome: random mutagenesis and RNAseq. EMS mutagenesis has been utilized since the very beginning of *C. elegans* genetic studies [1]. This technique results in the introduction of random point mutations throughout the genome of each cell of an exposed organism. If that cell should be a germ cell, and that germ should give rise to an offspring, the resulting animal will be heterozygous for the germ cell's point mutations, and these mutations may homozygose in some animals of the subsequent generation (one of the advantages of working with a hermaphroditic species – a gonochoristic genetic model would require at least another generation).

RNAseq is a relatively new technique that allows for simultaneous abundance measurements of all RNA transcripts in an organism, or a subset of those transcripts [2]. This technique, which relies on modern high-throughput sequencing technology, has been used to analyze nematode transcriptomes in the past [3].

Random mutagenic screen

As is standard practice in mutagenic screens, a parental population of worms (P0) was exposed to EMS and allowed to recover and produce progeny. These progeny (F1), which are potentially heterozygous for many mutations, were then distributed to new plates and allowed to develop. The second generation of progeny (F2) from the mutagenesis, which are potentially homozygous for some mutations, were then redistributed to fresh plates at one worm per plate.

In order to rapidly assess many thousands of potential mutants, a more efficient chemotaxis assay needed to be devised than was used previously in chapter 2. To this end, both the collection and testing of pheromone were optimized for high throughput. Instead of carefully selecting out worms for pheromone extraction, the progeny of the F2 population were rinsed off their plates, and pheromone was extracted from many strains in bulk in 96-well plates.

To speed the testing of pheromone, males were challenged with a modified chemotaxis assay containing worm water samples from sixteen F2 plates (Fig. 1). Since the aging of nematodes is time consuming, and finding a single negative in a background of positives is more difficult than the reverse, this screen was only used to search for mutant that precociously produced pheromone in young adulthood.

Through the course of this screen, we generated approximately 7,000 F2 clonal populations for testing. After filtering these strains for those suitable for high-throughput pheromone extraction (fast-growing, large brood size), 2,000 strains were chosen for testing. Testing ultimately

identified 28 candidate mutant strains, although none of these strains still produced a phenotype after retesting with the more strict protocol described in chapter 2. At the very least, we are satisfied that this assay has a false positive rate of less than 1.5%.

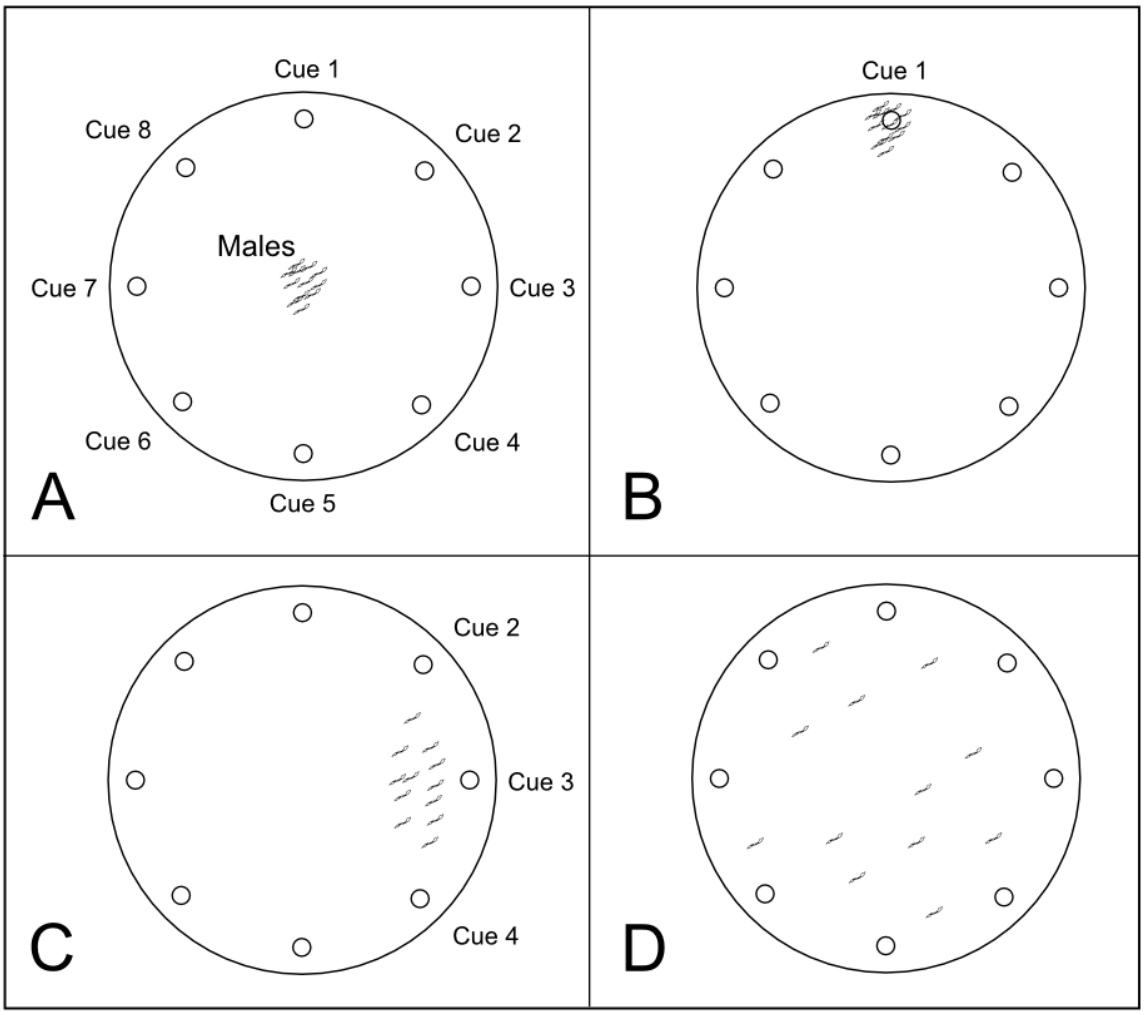

Figure 1: A. High-throughput assay diagram. Eight pheromone samples were placed circumferentially on the inside lid of the plate. Approximately fifty males were placed in the center of the agar. **B.** Ideal situation – All males have congregated beneath a single sample, cue 1, which will selected for retesting. **C.** Second best situation – Males have congregated in the general area of several samples, cues 2-4, which will all be selected for retesting. **D.** Worst case scenario – Males have positioned themselves randomly across the plate, appearing to favor no position. All eight samples will be discarded.

RNA-Seq

The goal of this experiment was to measure the abundance of all mRNA molecules in a population of nematodes to identify those associated with pheromone production. Choosing a single population of worms would produce too many potential genes of interest, so we had to sample from multiple distinct types of populations and use Boolean methods to whittle the list of candidates down to a manageable number.

We ultimately obtained sequencing data for four populations: young adult N2 (no pheromone), aged adult N2 (pheromone), young adult female mutants (pheromone), and old adult female mutants (pheromone). This approach would allow us to follow the effects of sperm depletion through two distinct mechanisms, as well as filter out transcriptional changes that occur merely as a result of age (fig.2).

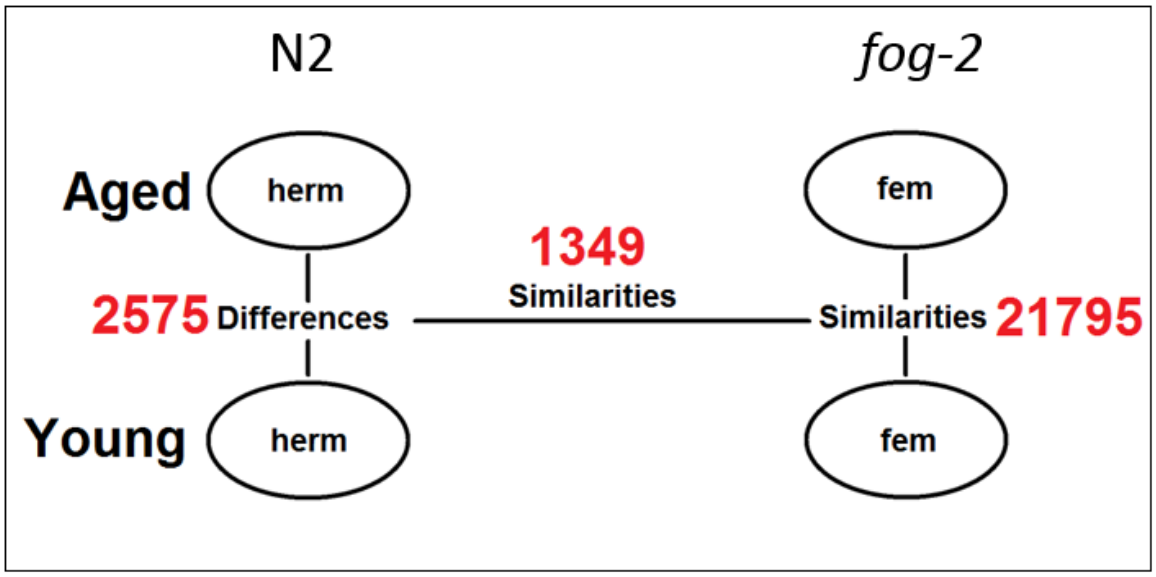

Figure 2: Diagram of RNA-seq scheme and summary of results.

Three biological replicates of each population were sequenced. All twelve samples were PCA plotted to ensure that the variance between individual samples was primarily caused by differences between populations rather than between replicates (fig. 3).

The overall results of this experiment reveal many genes that may be involved in pheromone regulation, and also validate the use of multiple comparisons. In the N2 set, over 2,500 genes undergo differential regulation as pheromone status changes, whereas in the mutant female set, over 20,000 genes remain steady as pheromone status goes unchanged. Fortunately, the intersection of these two groups of genes produces a more modest 1,349 candidates.

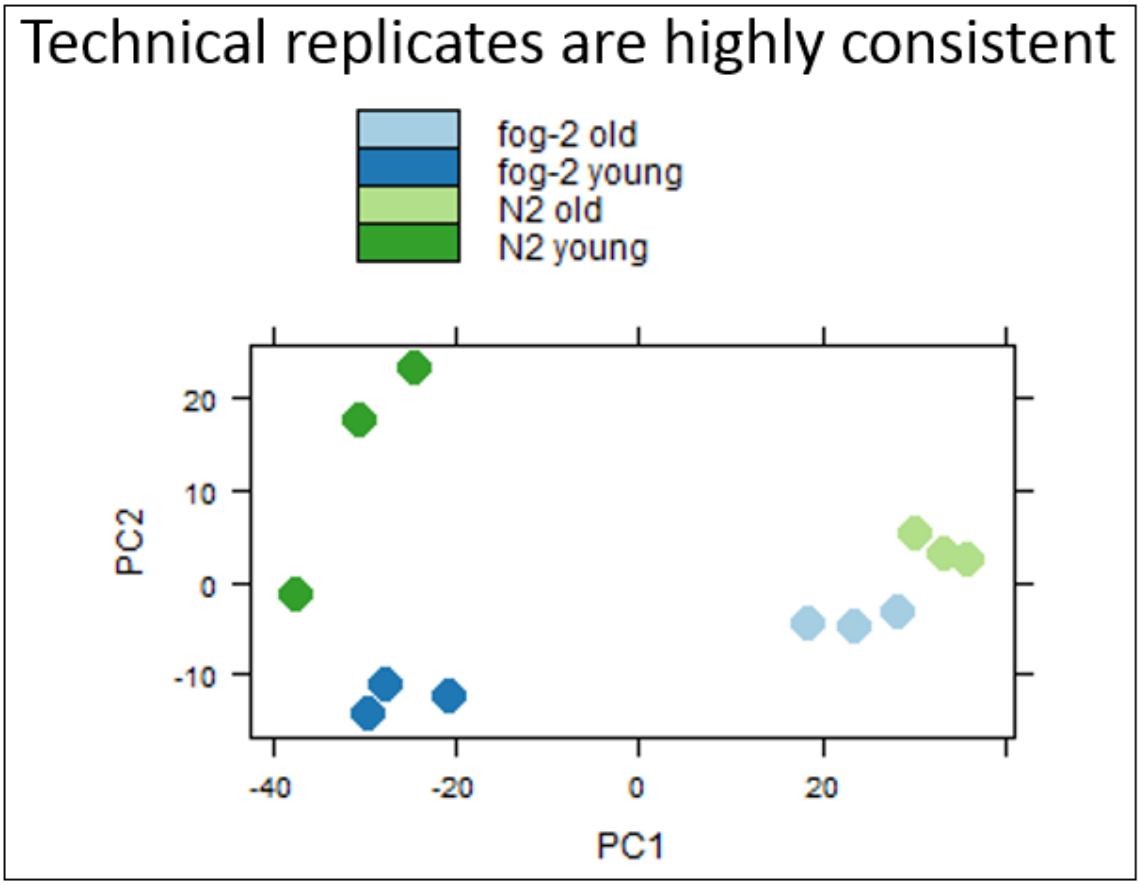

Figure 3: PCA plot of all twelve sequenced samples.

Although better than 2,500 or 20,000, 1,349 genes is still too many to assay in a single thesis project. Instead, this list of candidates was further filtered down to those in which expression levels were similar between the mutant female group and the aged N2 group, bringing the total number of candidates down to 700. This was then further filtered by selecting only those genes in gene classes considered likely to code for proteins that synthesize small molecules or regulate their production (fig. 4).

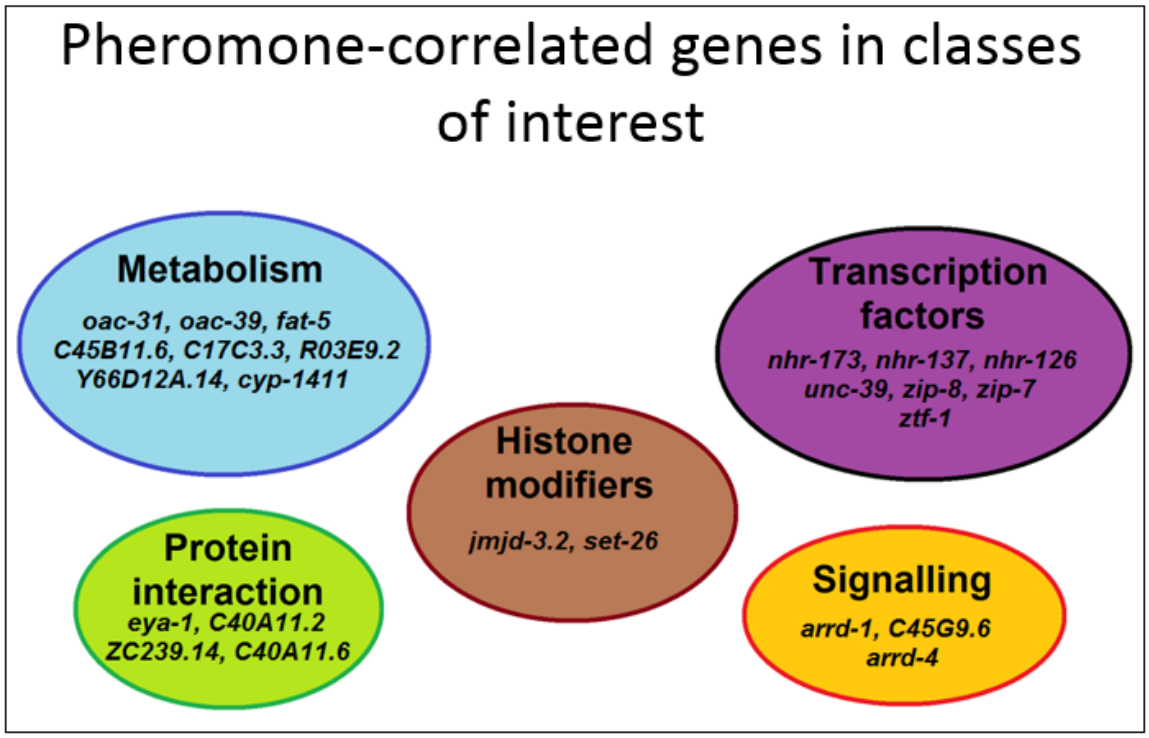

Figure 3: Genes of interest organized by gene class. For most genes, class was determined by sequence homology.

After this final filtering step, we arrived at 24 candidate genes we considered most likely to encode either regulatory proteins that govern pheromone production, or metabolic enzymes that actually generate the pheromone. Putative null mutations existed for ten of these genes, which were tested for pheromone activity. Seven of these genes are highly expressed in pheromone-positive samples, and were tested for an absence of pheromone production in old age (fig. 4). The other three were highly expressed in pheromone-negative samples, and were tested for precocious pheromone production (fig. 5).

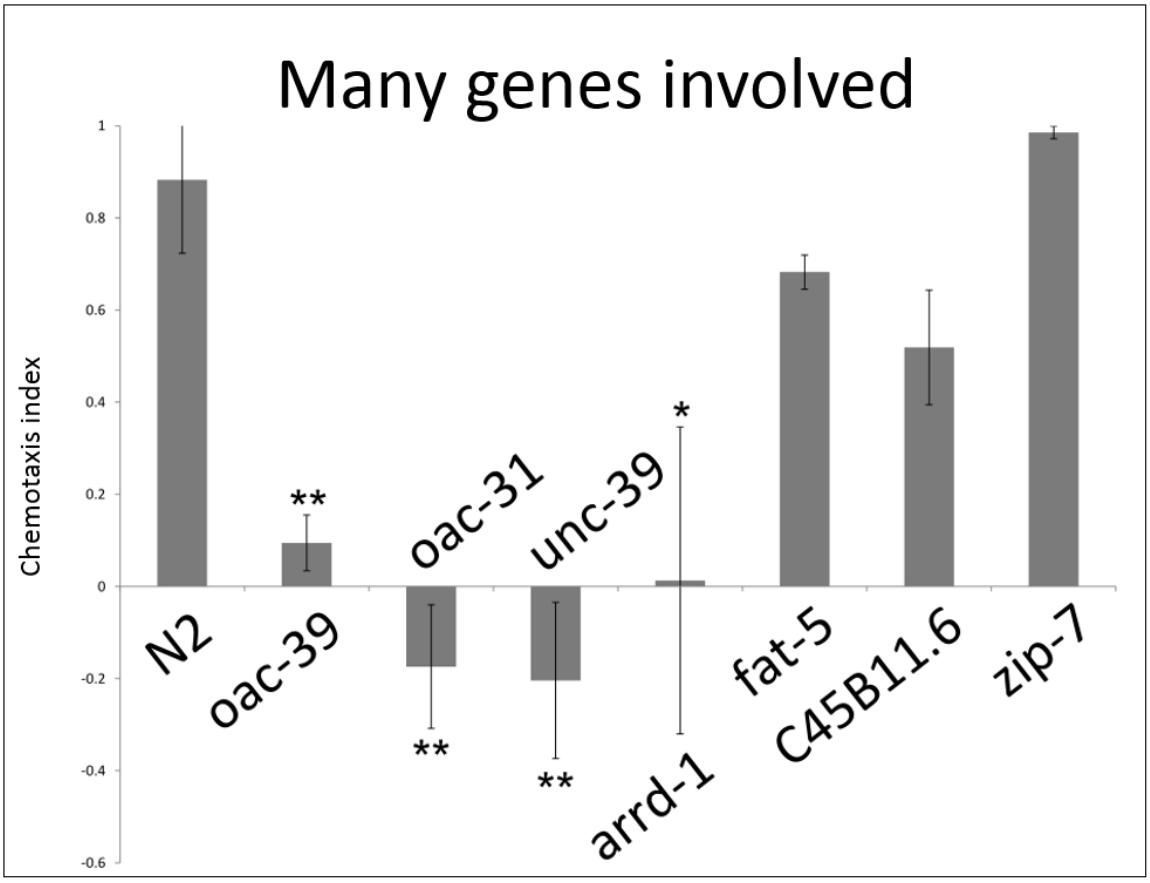

Figure 4: Attraction of males to aged hermaphrodites of N2 and seven mutant strains.

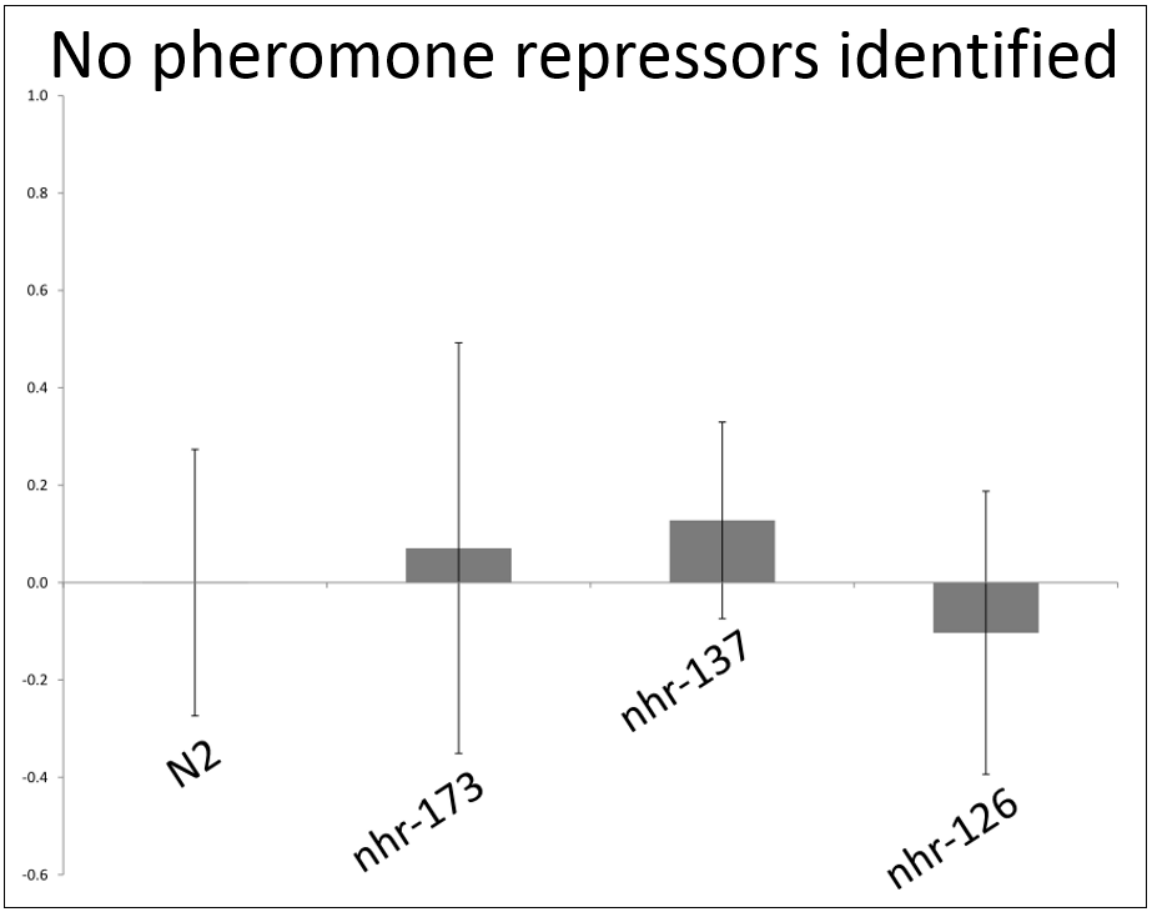

Figure 5: Lack of attraction of males to young hermaphrodites of N2 and three mutant strains.

From our testing of 10 genetic mutations, we found four genes that appear to produce the appropriate phenotype, although the result of *unc-39* turned out to be spurious, as outcrossing the mutant strain 3X to N2 eliminated the phenotype. Without any one of the three remaining candidates, adult hermaphrodites appear incapable of transitioning to a pheromone-positive state.

Very little is known about these three genes, *oac-31*, *oac-39*, and *arrd-1*. None have been studied directly in great detail, and most of what we know comes only from protein homology. The genes *oac-31* and *oac-39* are believed to encode o-acyltransferases, a class of metabolic enzymes. The gene *arrd-1* is believed to encode an arrestin, a class of proteins that inhibit the activity of G-protein coupled receptors.

Discussion

This project has identified multiple genes that may be involved in either the biochemical or regulatory pathway that underlies production of the unknown nematode mating pheromone. Further work is required for the validation of these results, but it is striking that three candidates were found from a screen of only ten strains. If these are merely false positives caused by background mutations, and not by fortuitously good dataset filtering, this would imply at least that there are so many genes that effect pheromone production you can find mutants by accident at a high rate.

If and when these candidates are validated as being involved in pheromone production, it will be helpful to assay these genes for site of expression and transcriptional interdependence, in order to help place these genes into a pathway, perhaps even in the context of *egg-3*.

At this moment in time, there is currently too little uncovered to draw firm conclusions about how these three genes influence pheromone production.

1. Brenner S: **THE GENETICS OF CAENORHABDITIS ELEGANS**. *Genetics* 1974, **77**:71-94.
2. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B: **Mapping and quantifying mammalian transcriptomes by RNA-Seq**. *Nat Meth* 2008, **5**:621-628.
3. Hillier LW, Reinke V, Green P, Hirst M, Marra MA, Waterston RH: **Massively parallel sequencing of the polyadenylated transcriptome of C. elegans**. *Genome Research* 2009, **19**:657-666.

Chapter 4:

Conclusions

At my last committee meeting, Professor David Anderson asked me what the one most important thing I discovered was. I feel torn between two statements: 1) Hermaphrodites simply behave like females with sperm, until they run out; and 2) Hermaphrodites can tell if their eggs are being fertilized.

Regarding the first statement, it has long been known that young hermaphrodites and young females of closely related species of nematode do not behave the same. As discussed in chapter 1, this has classically been interpreted as an evolutionary response to the rise of hermaphroditism – since hermaphrodites no longer require mates, those behaviors that lead to mating have been lost (either through actively negative selection, or simply drift and neutral selection). However, I hope I have demonstrated to the reader, through both a thorough examination of the literature and novel experiments, that this is most certainly not the case. Many authors have recognized that sperm replete females behave in a similar fashion to hermaphrodites, but very few appreciated that sperm deplete hermaphrodites behave like females! The behavioral scheme is essentially the same for both genders, but they merely begin in different physiological states.

In terms of actual new discoveries, however, I consider my most important to be that hermaphrodites can sense the fertilization of their eggs, or at least the simultaneous presence of mature oocyte and mature sperm in the spermatheca. As mentioned previously, this is a finding unprecedented outside of mammals. *Caenorhabditis* nematodes already possess a robust sperm detection system to prevent wasteful ovulation, so what could be the purpose of a second sperm or fertilization sensation pathway? Why would a nematode begin ovulating after a successful insemination, but only stop seeking mates when the sperm has proven effective?

The most obvious conclusion I can imagine is that scenarios exist in the wild in which a female or hermaphrodite may find herself inseminated with ineffective sperm. Perhaps this is a result of mating to sick or damaged males, or to males of a closely related but non-interfertile species. Alternatively, perhaps this is simply how it evolved – both ovulation and mate finding benefited from being linked to sperm status, and this arose through independent mechanisms for no particular reason except that it did.

Nevertheless, I believe this is a valuable approach to dissecting the communication of nematodes. While the careful study of nematode responses to pheromone exposure provides valuable information, analysis of this data can be difficult. Laboratory worms are grown in an environment that does not remotely resemble their wild environment – they are generally unexposed to pathogens and parasites, they eat food they are unlikely to encounter naturally, the temperature is held roughly constant, and they can only move in two dimensions! Although some behaviors are easy to make sense of (mating, dauer), for many others, it is unclear what the worms are actually trying to do. And this does not even take into account the possibility that a worm may benefit from *not* responding to a signal the way its sender intended.

But studying instead how a signal is generated and what physiological triggers are needed to release it, may reveal the purpose of a signal with great clarity.