

PHEROMONES IN FREE-LIVING AND PARASITIC NEMATODES

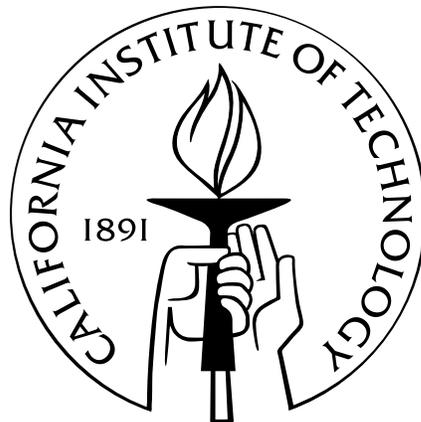
Thesis by

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In Partial Fulfillment of the Requirement for the

Degree of

Doctor of Philosophy



CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2012

(Defended June 17, 2011)

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Acknowledgements

To begin with, I must dedicate this thesis to several people that have helped to define my personal beliefs and perspectives:

1) My mother, Jennie, who showed me inspiration 2) My father, Gregory, who showed me discipline 3) My brother, Daniel, who jedi-mind tricked me into becoming fearless 4) Dima Kogan, for making the journey the destination 5) Paul Sternberg, for making science an adventure.

Before meeting Paul Sternberg, my friends told me that I simply *must* rotate in his lab. I can see why, given that he is probably one of the very few people that understand the way that I think about science. I had not gone into this thinking about a career, but rather “how can I get Paul to pay for this thing I need to study this other cool thing?” This is a man that will not bat an eyelash in the face of curiosity-driven science. Having said that, he will be the first to tell you to redo an experiment before turning it into a real project. I am not the first, nor will I be the last, to say that I could never imagine doing graduate work in any other lab.

I must thank the members of my committee for their insightful discussions and collaborations, without which I could not enjoy the multidisciplinary perspectives that have driven this thesis forward.

I have so many members of the Sternberg lab to thank. In retrospect, my bay-mates were extraordinarily invaluable to my daily thought process. For this reason, I have to thank Steven Kuntz and Ali Mortazavi for enthusiastically talking about anything and everything from worm genomes to British TV shows about evil butlers. I remember when I was having contamination issues with my bacterial cultures, Steven would willingly sniff the flasks and we would discuss the subtleties of its odor like two wine snobs. Of course I must thank Oren Schaedel for his support throughout the years. I have never argued with anyone as much as I have argued with Oren and yet I can always count on him for pretty much anything. I must thank Adler Dillman, one of the last remaining field biologists, for shamelessly loving science. Lastly, I must thank Dmitriy Kogan for creating the automated worm detection software that most of my thesis has relied upon.

Preface

As far as I can remember, I have always been curious about behavior, but several particular influences come to mind when I think about how the interest *really* developed. One such influence comes from John Steinbeck's novel, *East of Eden*. When it was adapted for Elia Kazan's film in 1955, several key components of the novel seemed to be left out; most notably the servant character, Lee, whose personal philosophies laid the foundation for the central theme: control of instinct. Lee offers the doctrine that humans have choice to alter their innate fears and desires, contrary to the protagonist Adam's deterministic belief that we are born with rigid instincts. Such is an important lesson for his son Cal, whose impulse to wreak havoc is appended by the profound realization that he can simply choose to reject the need to destroy things. I still have a vivid memory of the scene in which James Dean, who plays Cal, clutches his curled fist with conflict.

I often think about the tug-of-war between nature vs. nurture and the emotional drives that we can/cannot choose to follow or ignore. I think about the correlations between aggression/passivity and specific genes and sizes of structures in our brains, leading me to further wonder how much of our behavior is predetermined. I think about the infamous serial killer Ted Bundy, who claimed, "I just couldn't help it" and wonder if there is some truth to that.

I've also spent a great deal of my life working with children with developmental disabilities, witnessing the subtle ways that autistic individuals can sabotage their relationships by prioritizing specific needs over friendship. Sometimes those needs are to rock back and forth 10 times before answering the friendly question, "Do you want to play?", increasing the frequency of this rocking pattern when the other has finally left from insult. When I look at their expression, it seems to read: "I don't have a choice."

Finally I come to the worm. People often ask me why I chose to study the worm when it seems that my interests are so strongly aligned with human pathologies and disabilities. I disagree that the two are mutually exclusive, as I have felt no greater gratification than poking and prodding at one of Earth's most successful creatures. I am not interested in the worm such that I may study humans; rather I am interested in worms because they are interesting to study life. There exists a broader forum for the questions that I seek to answer; questions that feel the weight of a more diverse perspective. How does instinct evolve? What changes behavior? What *is* behavior? It is no coincidence that I have migrated from research of the mouse, to the fly, to the worm. With less, we can find more, and I believe that the work that is presented here will begin to reveal that there is indeed much, much more.

Abstract

Nematodes are among the most diverse phyla of animals, occupying almost every ecological niche available[1]. Their ubiquity has led to a number of problems for civilization, including the loss of crops and the spread of neglected tropical diseases. Because they are responsible for a broad range of agricultural and human diseases, many pheromone-mediated nematode behaviors have been described but very few pheromones have been identified.

We report, via high-performance liquid chromatography electrospray ionization mass spectrometry, the discovery that many free-living and parasitic nematodes secrete small-molecule pheromones called ascarosides. These pheromones, called ascarosides, were first found to play a role in sex attraction and induction into a stress-resistant diapausal life stage in the free-living organism, *Caenorhabditis elegans*. We have performed a double-blind purification of the female sex pheromone in the sour paste nematode *Panagrellus redivivus* and report that the female sex pheromone is composed of at least two ascarosides. We have also found that both free-living and parasitic nematodes respond to different concentrations of ascarosides through attraction or repulsion, demonstrating cross-species communication. These results suggest that ascarosides could be a universal nematode cue, similar to the role of N-Acyl homoserine lactones in bacteria quorum sensing.

Because ascarosides are nonvolatile, they can only mediate close-range communication. Nematodes have a well-characterized capacity for long-range chemoattraction to a range of

volatile cues. However, no studies have been done towards characterizing natural volatile cues derived from nematodes. Here I describe the discovery of volatile cues are produced by male-female species in the genus *Caenorhabditis*, but are lacking in the hermaphroditic species *C. elegans*, *C. briggsae*, and *C. sp11*. These volatile cues attract males (and sometimes females) from other *Caenorhabditis* species, demonstrating a cross-species gonochoristic cue.

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CHAPTER 1

Introduction

Evolution and Ecology

Nematodes (roundworms) occupy virtually every ecological niche available [1], making it difficult to classify them by lifestyle. They are found to inhabit sulfurous sediment, volcanic ash, deep-sea trenches, human lymph nodes, pig intestines, plant roots, whale placenta, arctic ice, and many other diverse ecosystems [2-7]. Despite their diversity, nematologists have cast a general scientific divide between parasitic (plant, vertebrate, invertebrate) and free-living (soil, fresh, marine, brackish, or estuarine water) nematodes* [8]. This divide has led to differences in nomenclature (e.g. larval vs. juvenile), creating the illusion that free-living and parasitic nematodes have very different life cycles, when in reality all nematodes have five main life stages, partitioned by four molts [9].

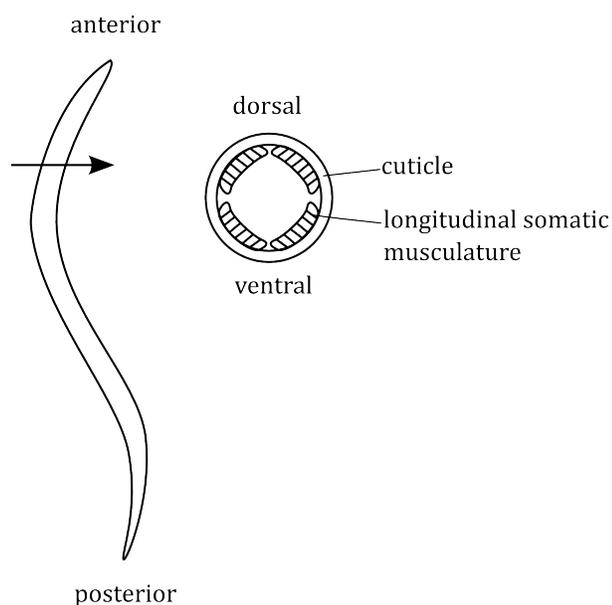


Figure 1: Nematodes have a remarkably uniform body plan. The dorsal and ventral muscles oppose each other in contraction, creating the nematode's trademark wave-like movement. Adapted from Croll (1970)[10].

* However, many nematode species may occupy both free-living and parasitic lifestyles during different parts of their life cycle.

Another common trait among nematodes is their similar body plan (see Figure 1), despite the wide range of body size, ranging from 0.3 millimeters to 8.4 meters[†] in length [11]. The typical nematode has a pseudocoelomate, cylindrical body that tapers at both ends [12] and an intestinal, reproductive, endocrine, and nervous system [13] held together by hydrostatic pressure. Because nematodes lack a true coelom, traditional tree topologies position them in the ancestral position when compared to two other major model systems, the mouse and the fly (Figure 2a) [14]. Alternatively, nematodes have been clustered with arthropods for their shared ability to molt (Figure 2b) while others predict that they may actually be closer to vertebrates (Figure 2c) [14].

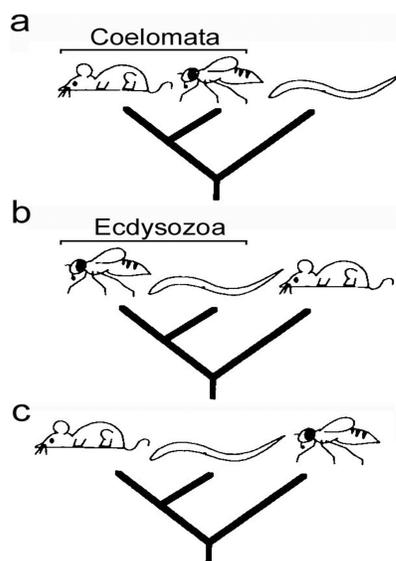


Figure 2: Comparison of three major model organisms: *Mus musculus* represents “the vertebrate”, *Drosophila melanogaster* represents “the arthropod”, and *Caenorhabditis elegans* represents “the invertebrate”. Model (a), which places *C. elegans* as the outgroup, is favored by the molecular data, including sequence comparison of RNA polII and III, mitochondrial rDNA, cytochrome c, and 18S rRNA[14]. Taken from Fitch, D.H.A. (2005) *WormBook*[14].

[†] The appropriately named *Placentonema gigantissima* is an 8-meter-long parasitic nematode that dwells within the placenta of sperm whales.

The representative nematode for these comparative analyses is the soil-dwelling *Caenorhabditis elegans*; which is the first metazoan to have its full-genome sequenced [15]. Comparative analysis between *C. elegans* and other nematodes have proven to be important, given that nematodes are incredibly diverse within their phylum and can offer much insight into how specific changes in molecular function and development arise [14]. In fact, very small differences in their body anatomy has led to the successful invasion of many different habitats [16]. For example, nematodes from the order *Enoplina* have developed setae (bristle-like protrusions) on their head, which allow worms to attach to a surface and prevent backslide during peristaltic propulsion [17] (see Figure 3A and 3C). Punctations found in the cuticle of nematodes from the order *Chromadorida* (see Figure 1F) are tentatively classified as ornamentations, however some speculate that these may actually be canals that run through the cuticle for a yet undefined function (Baldwin, unpublished).

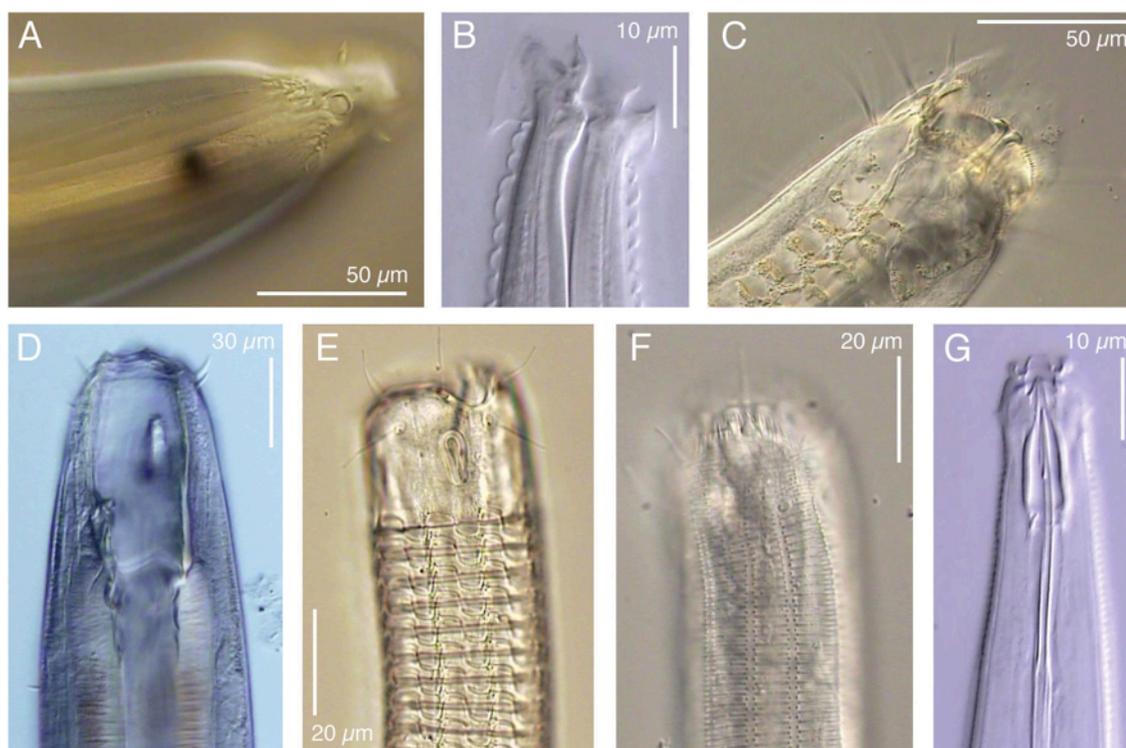


Figure 3: Example of divergence of nematode anterior morphology

A) *Thoracostoma* sp (Enoplina). **B)** *Acromoldavicus mojavicus* (Tylenchina: Cephalobomorpha). **C)** *Enoploides* sp. (Enoplina). **D)** *Pontonema* cf. *parpapilliferum* (Oncholaimina). **E)** *Ceramonema* sp. (Plectida). **F)** *Latronema* sp. (Chromadorida). **G)** *Actinca irmae* (Dorylaimida). Taken from De Ley, P. (2006) *WormBook*[18].

The marine nematode *Oncholaimus campylocercoides* is able to survive their anoxic, sulfidic environment by developing S-8 rings and polysulfur chains in their epidermis (which disappear upon reintroduction of oxygen) [19]. These are just a few of many morphological adaptations that have helped to establish the ubiquity that nematodes enjoy. Nematodes also have a wide range of behaviors which include chemotaxis, food-seeking, host-seeking, mate-finding, mating, swarming, dispersal [8], nictation (this is otherwise best described as “flailing”), stress-resistance, response to mechanical stimuli, egg-laying[‡],

[‡] Female *Mermis nigrescens* ascend from the soil and climb up vegetation towards the light before engaging in egg-laying. When placed in the dark, the female ceases egg-laying and only resumes with re-illumination.

response to electric currents, response to gravity, response to light, and learning [20].

Variation in these aforementioned behaviors is important for successful adaption to different hosts and/or environments. Such behaviors can even be specific to certain life stages.



Figure 4: Leaping behavior of *Steinernema carpocapsae* towards insect host *S. carpocapsae*, during its infective juvenile life stage, is often found standing on its tail and waving its head side to side. This is thought to help them sample odors from the air in order to detect passing insect hosts. When an insect host is within proximity, they bend into a loop and fling towards the host [21].

For example, many nematodes have a stress-induced alternative life stage in which their body cavity becomes sealed off to the external environment, allowing them to survive desiccation. This life stage is often associated with nictation, a behavior in which the nematodes can balance on their tail and wave their heads back and forth, presumably to attach onto a passing animal that may transport it towards more favorable conditions (more

food, less population density, change in pH, etc.). In the insect-parasitic nematode, *Steinernema carpocapsae*, this behavior is extended such that they may leap towards passing hosts at a distance that is nine times its body length [22] (see Figure 4). Leaping is not seen in all insect-parasitic nematodes, nor do all nematodes from the genus *Steinernema* have this ability, making it a particularly interesting trait for studying the evolution of behavior (Dillman, personal communication).

There are many combinations of behavioral variants that help nematodes adapt to their given environment, many of which we still do not understand. It is only with a combined look at ecology and evolution that we might begin to appreciate the complexity of nematode behavior.

The Study of Behavior

Animal behavior is traditionally described as the integration of intrinsic and extrinsic inputs, which are observed as set of actions elicited by a given organism (or set of organisms) [8]. The goal for any ethologist is to understand what this actually means, requiring the careful investigation of precise and measurable stimuli, as well as mechanisms underlying an individual's response. Let's take, for example, one of the landmark experiments that helped to establish the field of ethology in the 1930s; the study of territorial fighting behavior in three-spined sticklebacks (*Gasterosteus aculeatus*). During the spring mating season, males develop a red coloration on their bellies and defend their territories by fighting off any other males of their species, which they recognize by their red underbelly [23]. This behavior is interesting because of its specificity to a

developmental life stage (redness only occurs in adult males) and clear motive (territorialism specific to mating season).

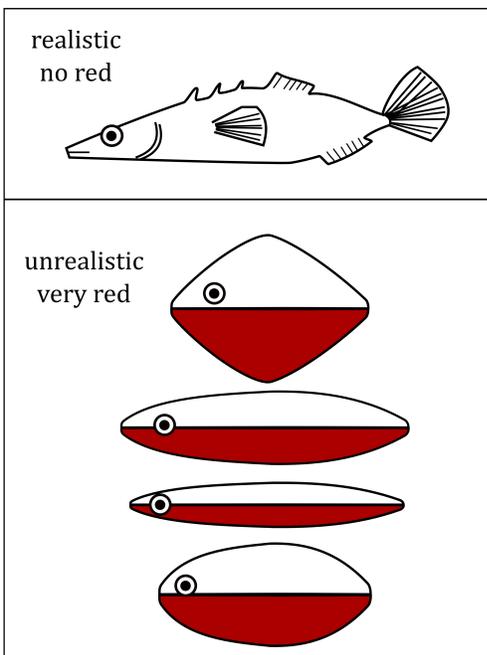


Figure 5: To identify specific triggers for aggression in male sticklebacks, Tinbergen created several models with different traits and presented them to males. Some were realistic replicas of the male stickleback, but lacked any red coloration, whereas several other models lacked many of the characteristics of a fish, but had red underbellies. Adapted from Tinbergen (1948)[23].

It was with these experiments that Nikolaas Tinbergen demonstrated the concept of fixed action patterns (FAP). FAPs are sequences of instinctive behaviors that are observed when an individual is presented with a “releaser”[§] stimulus [24]. Because FAPs occur without prior exposure or training, they are one of a few types of behaviors that are considered instinctive. In this study, Tinbergen made several models of sticklebacks and presented them to live male sticklebacks (see Figure 5). He found that the male sticklebacks ignored the realistic model lacking a red underbelly and attacked the unrealistic red-bellied models,

[§] It was thought that specific stimuli would “release” a set of behaviors that are constantly suppressed and thus require an unlocking mechanism.

proving that the sticklebacks react only to the red and neglect most other characteristics [25]. As with any discovery, this conclusion led to more questions. How did this behavior evolve? Can sticklebacks only detect a narrow range on the visible spectra, or are there special receptors for longer wavelengths that trigger a response to the aggression center of the brain? Do males still behave this way when there are a plethora of females and viable nesting sites? Because the study of this behavior involves investigation from many angles, Tinbergen defined a set of questions that should be asked of every animal behavior [26]:

1. **Causation** (mechanism): What are the stimuli that elicit the response?
2. **Development** (ontogeny): How does the behavior change with age?
3. **Evolution** (phylogeny): How does the behavior compare with similar behavior in related species and how might it have arisen through the process of phylogeny?
4. **Function** (adaptation): How does the behavior impact survival and reproduction?

I firmly believe these are the bare minimum number of tenets to consider when studying any behavior. These are the tenets that I will continue to address, towards the goal of revealing multidisciplinary perspectives throughout the investigation of pheromone-mediated nematode behavior.

Nematode Response to Chemicals

“Obviously mononchs (predatory nematodes) hunt by the aid of some sense other than sight...picture these ferocious little mononchs engaged in a ruthless chase in the midst of stygian darkness. We may imagine them taking up the scent of the various small animals upon which they feed... pursuing them with relentless zeal that knows no limit but repletion.” –Nathan A. Cobb, founder of Nematology (1917).

There are two main categories of nematode locomotive response to chemical cues: kinesis and taxis [10]. Kinesis describes movement that lacks directional orientation and is affected by the intensity of the stimuli. For example, if a photophobic nematode is exposed to light, it may simply increase its speed and return to its normal speed upon removal of light. In this situation, the nematode is not avoiding light in a directional manner but rather spending more time in optimal conditions. The other type of nematode locomotive behavior is taxis, which involves the directed orientation towards or away from the stimuli. Nematodes have several possible methods of responding to chemical gradients via taxis (see Figure 6) [27].

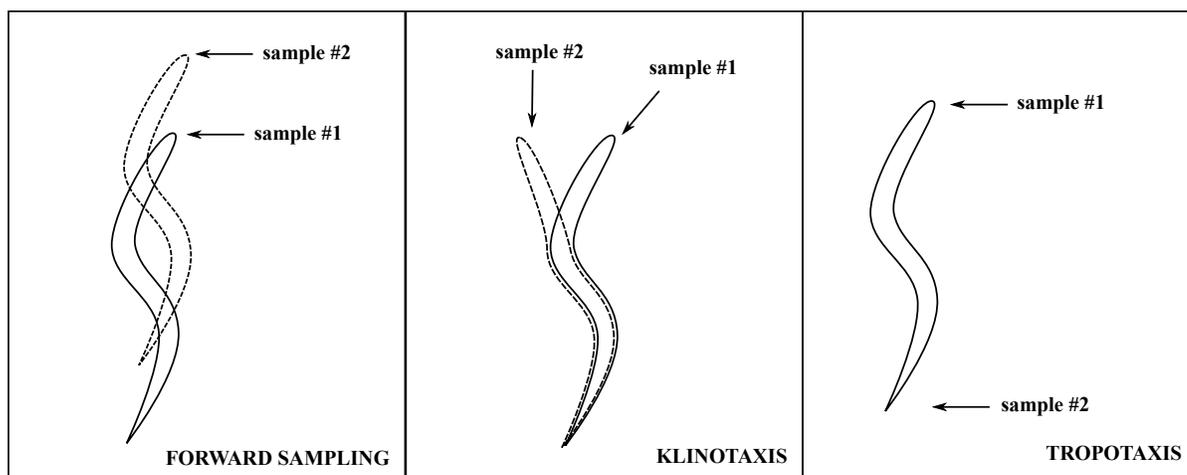


Figure 6: Nematodes can respond to chemical gradients (taxis) by forward sampling, klinotaxis, or tropotaxis [27].

In forward sampling, nematodes compare two different samples (and presumably different concentrations of a chemical) at different time intervals separated by forward movement. In klinotaxis, nematodes compare left and right samples in their environment with side-to-side movement of their receptors (in this example, the receptors are in their head). In tropotaxis, nematodes use more than one receptor in different locations of their body to sample from two different sources at the same time. Studies have demonstrated that klinotaxis is the most likely method of decision-making in *C. elegans* [28, 29]. *C. elegans* has several types of sense organs (which are largely conserved across nematodes): the anteriorly located paired amphids, inner and outer labial papillae, cephalic papillae, and the posteriorly located phasmids (see Figure 7) [30, 31]. Studies in a number of species have shown that the amphids are the primary chemosensory organ [32, 33]. They are the largest and most complex of the anterior sense organs and come in pairs, surrounding the nematode head region.

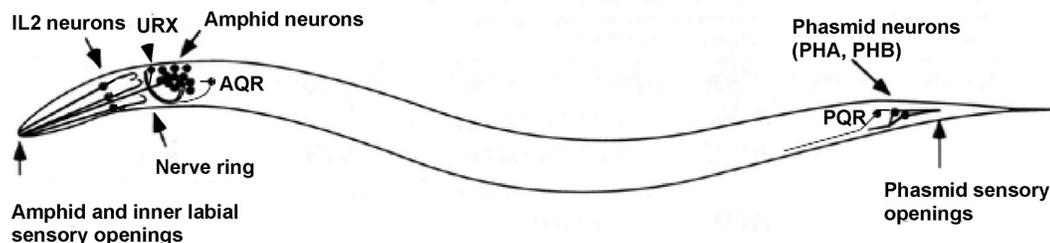


Figure 7: *C. elegans* sensory organs. Taken from Bargmann, C.I. (2006) *WormBook* [34].

The ability of *C. elegans* to respond to a wide variety of olfactory and gustatory cues is mirrored in the organization and structure of the amphid cilia, which are made up of both volatile and water-soluble receptors (see Figure 8) [34].

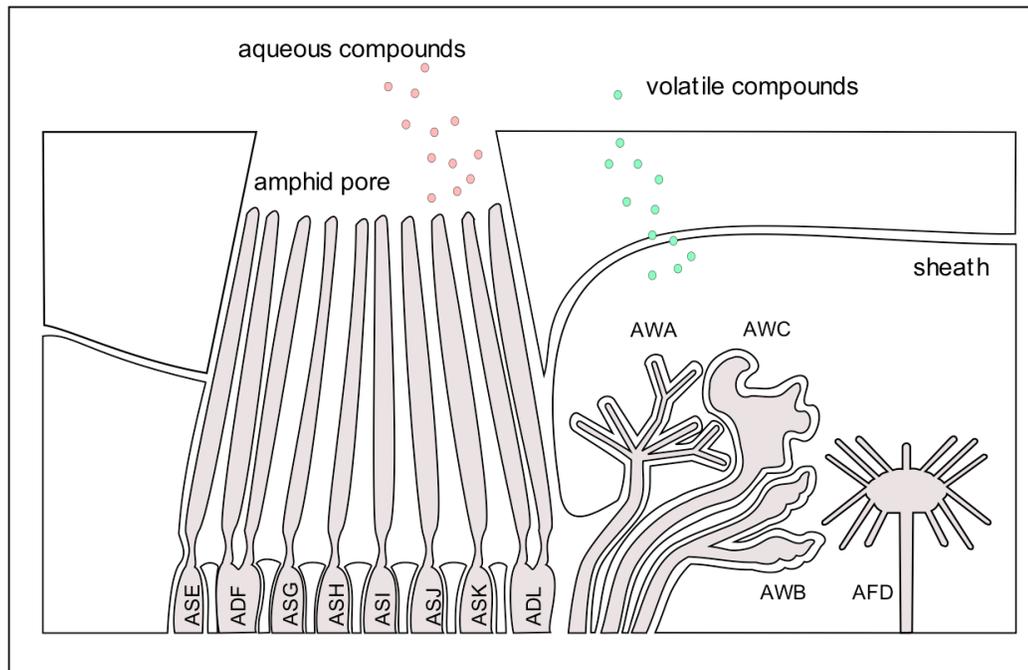


Figure 8: Closeup of amphid sensory openings in *C. elegans*. Adapted from Bargmann, C.I. (2006) [34].

C. elegans' amphid structure has eight pairs of dendritic processes that are exposed to the outer environment (gustatory) and four pairs of wing-shaped dendritic process that are buried beneath the sheath (volatile) and thus require gases to penetrate for exposure [35]. Because both types of chemical cues are important for long and close range chemotaxis, it is important to consider both volatile and nonvolatile cues in the investigation of nematode pheromones.

Nematode Pheromones

Pheromones are the most fundamental communicative cue for most organisms, possessing many advantages over other types of signals, such as their utility in darkness and energetic

efficiency (less than a microgram of a simple compound can produce a signal that can last for days) [36]. The word “pheromone” is limited to chemical interactions within species, although its general usage has implied otherwise. The correct nomenclature for chemical communication is as follows (see Figure 9):

semiochemicals (inter- and intra-specific interactions)	
pheromones (intra-specific interactions)	allelochemicals (inter-specific interactions)
sex pheromone response between or within sexes	allelomone → ⊖ negative response in recipient
epidietic pheromone regulate population densities	kairomone → ⊕ positive response in recipient
alarm pheromone warning or protective signals	synomone ⊕ → ⊕ positive response in both emitting and responding
	apneumone X → ○ signal produced by non-living matter

Figure 9: Classification of chemical communication signals [37-39].

Many studies have proven that nematode sex pheromones exist, but few have tested for the effects of these pheromones on other species [40]. Therefore, most nematode sex pheromones are limited to their intra-specific definition by default that attempts have not yet been made to prove otherwise. For example, the first pheromone discovered in *C. elegans* is the ascaroside, *ascr#1*, for its small role in the induction of the diapausal life stage known as dauer [41]. Although studies have shown that crude pheromone from *C.*

elegans can induce dauer formation in *C. briggsae*[41], no studies have been performed to demonstrate the utility of ascarosides in non-*C. elegans* dauer formation. This is surprising, given that there exist many examples of intraspecific chemical communication between (and within) vertebrate and invertebrate species[42-45] and is perhaps attributable to the convergence of interest in *C. elegans* throughout the past few decades. In fact, only one other pheromone has been identified from a nematode species other than *C. elegans*: vanillic acid (see Figure 10) is the female sex pheromone in the soybean cyst nematode, *Heterodera glycines*[46]. A significant amount of work has been done in this plant parasitic nematode, perhaps because it is responsible for a loss of ~ \$500 million annually[47].

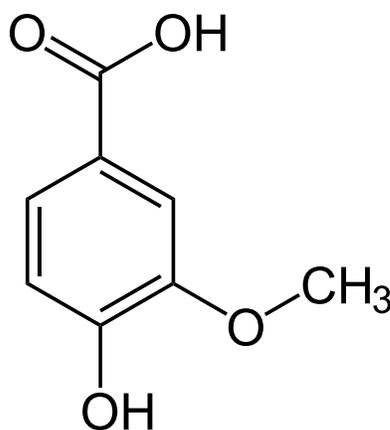


Figure 10: Female sex pheromone of *Heterodera glycines*: vanillic acid

Isolation of sex pheromones in *C. elegans* has led to the discovery that a blend of at least three ascarosides (see Figure 11) mediate mate finding[48]. It is surprising that ascarosides played a role in both mate finding and dauer formation[49], given that they are two very different survival strategies (see Figure 12). Recent work has also revealed that ascarosides

play a role in aggregation (see Figure 12) by attracting both *C. elegans* males and hermaphrodites to gather in groups for long periods of time (Srinivasan, unpublished).

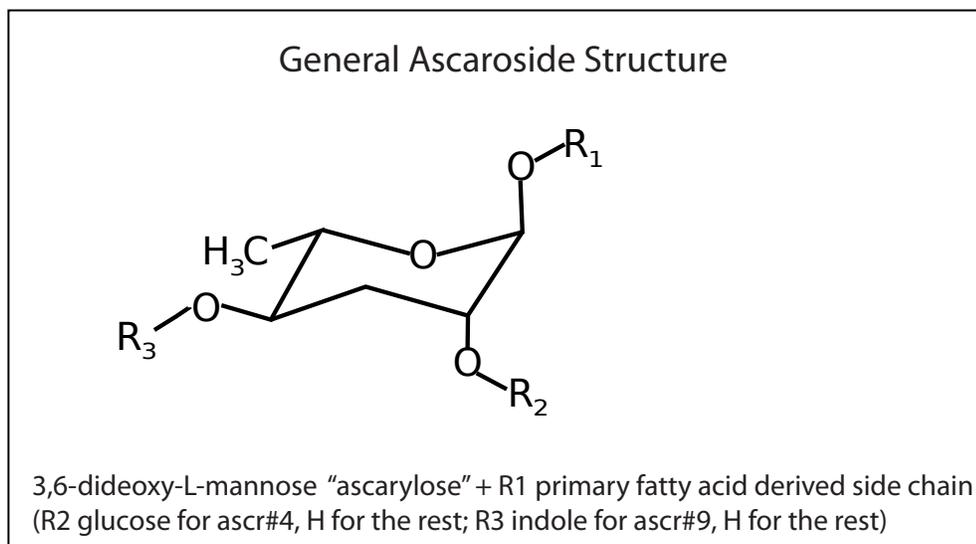


Figure 11: General structure of ascarosides: ascarylose sugar ring + modifications in lipid tail. Adapted from Edison (2009)[50].

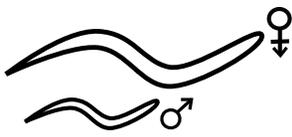
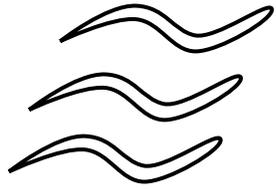
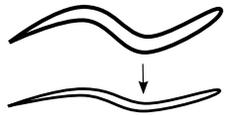
mate finding	aggregation	dauer formation
ascr#2 ascr#3 ascr#8  <p>Hermaphrodites produce these ascarosides to attract males</p>	icas#3 icas#5 icas#9  <p>These ascarosides induce aggregation of both males and hermaphrodites</p>	ascr#1 ascr#3 ascr#5 icas#9  <p>These ascarosides affect the percentage dauers formed in a population with unfavorable conditions</p>

Figure 12: Role of ascarosides in *C. elegans*: different ascarosides (and some overlapping) play a role in mate finding, aggregation (Srinivasan, unpublished), and induction into the diapausal life stage known as dauer [48, 49, 51].

This has led to the further investigation of *C. elegans* ascarosides, which are secreted in combinatorial blends that vary across different life stages[52]. Not much is known about the biosynthetic pathway for ascarosides, although it has been found that the signaling deficient *C. elegans* mutant, *daf-22*, fails to produce several ascarosides[48, 53]. *daf-22* was first discovered for the abnormal **dauer** formation phenotype observed in mutants[54]. It encodes the *C. elegans* ortholog of human sterol carrier protein SCP2, which catalyzes the final step of peroxisomal fatty acid beta-oxidation[55]. *daf-22* mutants are found to accumulate massive amounts of fatty acyl-coAs (up to 100-fold), causing severe developmental defects and abbreviated lifespan, suggesting that ascaroside biosynthesis is essential to *C. elegans* homeostasis and that the conversion of toxic long-chain fatty acids has provided a subset of readily-excreted pheromones that are then utilized for other purposes[56]. This would be interesting and would help to explain the conservation of ascarosides in other nematodes (as will be discussed later), given that the presence of these pheromones may be linked to conserved metabolic pathways that are conserved between nematodes. DAF-22 fusion protein tagged with GFP is expressed in the intestine, hypodermis, and body wall[55], lending weight to the theory that ascarosides are produced in the intestine and excreted through the mouth, anus, or excretory pore.

It is clear that ascarosides are important pheromones for several behaviors (and a symptom of good homeostatic balance) in the free-living nematode, *C. elegans*. However much work remains to be done on other nematodes, especially since there exist over 25,000 known nematode species[57]. Because investigation of both close and distant relatives to *C. elegans* would help to reveal general nematode mechanisms, I have begun my work with other nematodes, such as the sour paste nematode, *Panagrellus redivivus*. This approach

has contributed towards a greater understanding of nematode pheromones and cross-species nematode communication.

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CHAPTER 2

Source of the mate-finding pheromone in *Caenorhabditis elegans*

Abstract

Characterization of the nature of the sex pheromone in nematodes is important for understanding and potentially regulating populations of nematodes that affect parasitism, agriculture, and important ecologic processes. Because the free-living nematode *C. elegans* remains one of the best studied model organisms, it serves as a good starting point for addressing this subject. Previous studies in *C. elegans* have shown very little about the source of the mate-finding cue and about the processes that regulate its production and release. Also, there has yet to be any study on the volatile components of the mate-finding cue, which may be largely responsible for long-range attraction.

One way of revealing sources of the mating pheromone is to utilize a bioassay comparing attraction of males to secretions collected from wild-type, mutant, and laser-ablated hermaphrodites. These experiments will help identify necessary cellular components of the mating pheromone and possible mechanisms that help to regulate its synthesis and secretion.

Introduction

Over 30 species, but relatively few genera, of free-living, plant-parasitic, and animal-parasitic nematodes have been shown to exhibit pheromone-mediated behavior[1, 2]. Being the most abundant metazoan (by individual count) on earth, nematodes play a significant role in many important processes, such as infectious diseases, agricultural sustainability, and biogeochemical regeneration. Because the identification of mechanisms controlling nematode mating and growth carry such broad implications, it has been of great interest to characterize the nature of the mate-finding cue and how it affects populations across multiple genera. However, not much is known about the site of sex pheromone production in nematodes[3]. Possibilities might include secretions through the vulva from the gonad, excretions from the digestive tract or excretory pore, or simply from the cuticle to the environment directly.

A range of studies support the likelihood that female sex pheromones are produced by the gonad and exit via the vulva. Because ascarid sex organs are large enough to dissect, they have been tested in isolation and it has been reported that both male and females of *A. suum* are attracted to sexual organs from the opposite sex[4]. It has been suggested that the female pheromone in the mouse pinworm, *Aspicularis tetraptera*, is secreted from the glandular cells of the female pulvillus (part of the reproductive organ)[5]. Studies on the source of the female sex pheromone in the free-living nematode *Panagrellus silusiae* also suggest that the gonads are a likely source[6]. Males from the rodent parasitic nematode species, *Heligmosomoides polygyrus*, demonstrate sexual behavior by flaring their copulatory bursa in the presence of females. However, when the females were treated to prevent release from the vulval area, males failed to flare their copulatory bursa, suggesting

that the sexual cue is secreted from the female vulva [7].

Several studies have suggested an alternative source of the sex pheromone. Simon and Sternberg (2002) reported evidence of a sexually dimorphic mate-finding cue in *C. elegans*, yet they found that vulvaless mutants were as attractive as wild-type hermaphrodites, implying that the vulva is not necessary for the release of the cue. Chasnov et al. (2007) found conflicting results, reporting that *C. elegans* males failed to show significant attraction for hermaphrodites. Instead, they characterized a female-derived attractant in the closely related, male-female species, *Caenorhabditis remanei*. They found that the female somatic gonad is required for the production and secretion of *C. remanei* attractant, but that the vulva is not necessary for its release. I have long speculated that male-female cues are much stronger than hermaphroditic cues, given that male-female species must find each other in nature and hermaphrodites do not require males. This would explain the discrepancy between the Simon and Chasnov study (and support Simon's conclusion that a hermaphrodite cue exists), given that Chasnov et al. collected secretions at a concentration of 1 worm per 20 microliters of buffer, which may be sufficient for stronger cues, but may be hidden by dilution.

Here I consider the chemoattraction assays that have previously been used to study mate finding in *C. elegans* and describe the parameters that have influenced the development of my own bioassay. I also describe a series of experiments that suggest findings contrary to both previous experiments: I report that *C. elegans* hermaphrodites have a mate-finding cue and that the vulva is necessary for its release.

Results

There have been three independent studies on the mate-seeking behavior of *C. elegans* males to hermaphrodites, each of which have influenced the development of my own behavior assay and have contributed to my perspectives on nematode chemical signaling. I will discuss the first two in this chapter and describe the third in the following chapter.

1. Simon and Sternberg (2002)[8]: This study provided the first evidence of a mate-finding cue in the free-living nematode *C. elegans*. They describe a sexually dimorphic mate-finding cue produced by hermaphrodites (and not males) that attracted males (and not hermaphrodites).

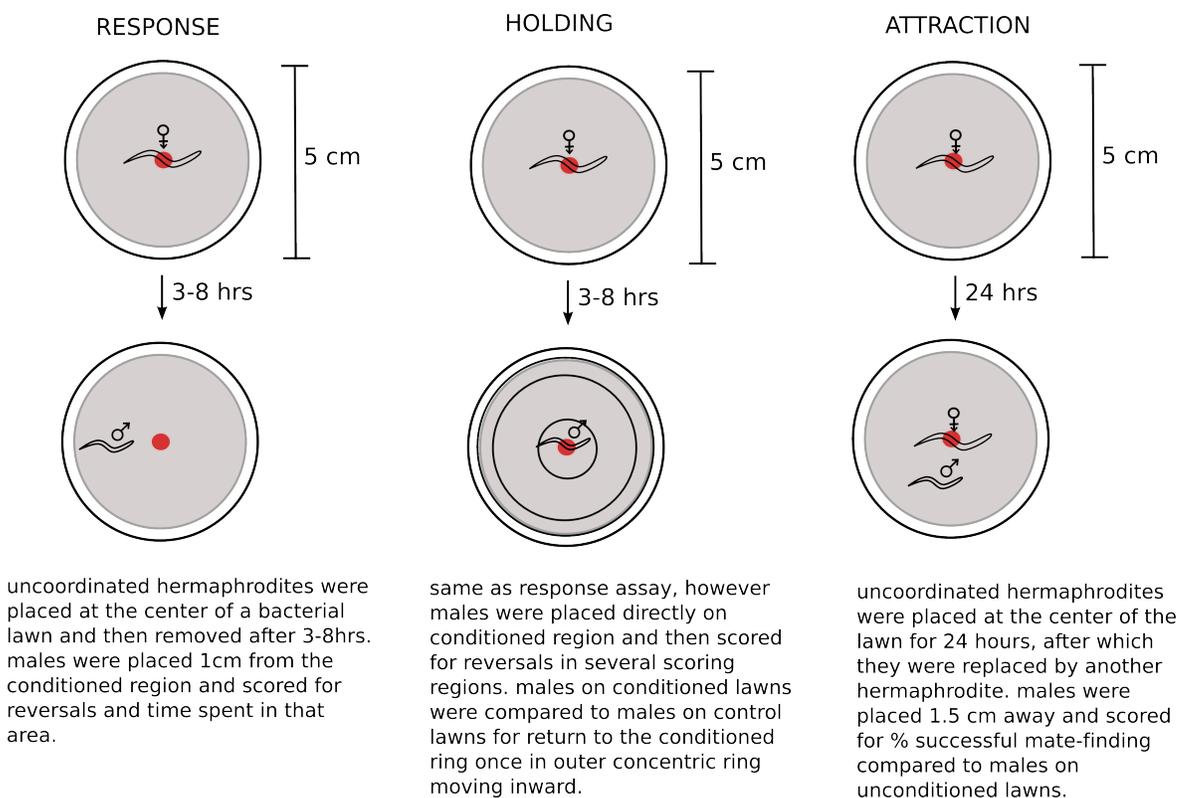


Figure 1: Simon and Sternberg (2002) Assays used to score for male chemoattraction to hermaphrodites[8]. (bacteria = gray) (cue = red dot)

They used several types of assays (see Figure 1), which utilized an uncoordinated mutant hermaphrodite, *unc-52*, to stay in one place on a bacterial lawn such that a male may be subsequently tested for response to the hermaphrodite-conditioned region. They found that males are attracted to, reverse direction of movement frequently, and remain in regions conditioned with hermaphrodites. Males were also observed to be more effective at finding their mates in a shorter amount of time on pre-conditioned lawns vs. unconditioned lawns. Next, they used vulvaless mutants, with strains containing *let-23* and *lin-3* mutations, to demonstrate that vulvaless mutants are as attractive as wild-type hermaphrodites. Although each individual was scored (via microscopic examination) for lack of a complete vulva, genetic mutants can never guarantee full removal of the affected organ or tissue. For this reason, I am skeptical from this evidence that the vulva fails to play a role in sex pheromone secretion. In an effort to reproduce these experiments, I used the same uncoordinated mutants and observed that the individuals tended to move > 1.5cm within 1 hour, thus I was unable to verify or disprove these findings by direct comparison.

2. Chasnov et al. (2007) [9]: This study reports that *C. elegans* does not have a cue, contrary to the Simon and Sternberg (2002) findings. In these experiments, worm secretions were collected from hermaphrodites (*C. elegans* and *C. briggsae*) and females (*C. remanei* and *C. brenneri*). Adult males were isolated and tested for attraction to the hermaphrodite or female cue collected from their own species. Chasnov et al. used several different bioassays, each utilizing a paralyzing agent (sodium azide) to hold males within a scoring region once they enter (see Figure 2). They found that hermaphrodite-derived cues fail to attract males from their own species whereas female cues attract all males, speculating that the two hermaphroditic species lost their ability to secrete a sex pheromone

via the simultaneous mutation of loss-of-cue and gain-of-sperm production. I find this to be a misleading conclusion, given that the assay had several design flaws (which actually lead to a very different, more interesting conclusion).

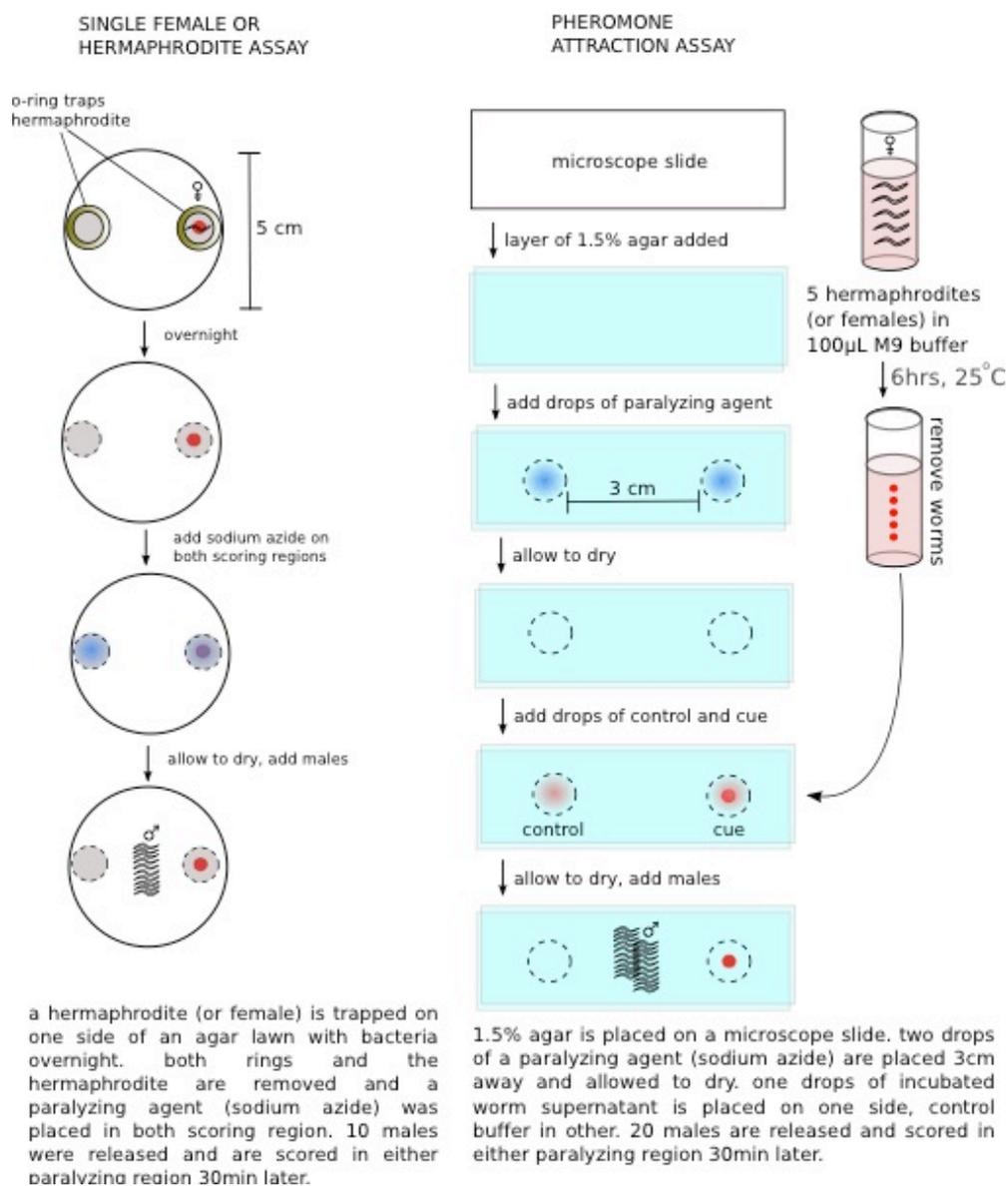


Figure 2: Chasnov et al. (2007) Assays used to score for male chemoattraction to hermaphrodites or females [9].

(bacteria = gray) (buffer = pink) (cue = red dot) (buffer + cue = pink + red dot) (sodium azide = dark blue)

The first problem is that the worm supernatant was collected at a concentration of 5 individuals per 100 μ L at 25°C. That is a very low concentration to collect from, given that each individual has about 15x their body volume in buffer that they are secreting into. Although I don't know enough about how effective dilute pheromone might be in nature, I would say that erring on the less concentrated end of the spectrum is not sufficient when making the claim that something does not exist. I would at the very least lyophilize the buffer to test a concentrated smaller volume before ruling out the existence of an attractant. Also most experiments with *C. elegans* are performed at 20°C, even though some speculate that their natural environment in compost might be closer to 25°C. Because we do not know enough about this matter, I cannot comment on whether this is better or worse for the design of this assay.

Secondly, these assays utilize a scoring method that prohibits individuals from sampling both scoring regions before deciding on a preference. Because sodium azide is combined with the control and cue region, a male may choose to enter one region or the other but cannot leave their first choice before they become paralyzed. This is a favorite method used for chemotaxis assays, as described by Bargmann and Mori (1997)[10]. However, this method only works if there is actually a chemical gradient. If a sufficient chemical gradient has not formed by the time that males are placed between the two regions, they would be forced to move in either direction without any information. An exception would exist in the presence of a volatile cue, which would provide information if sufficient amounts of volatile cue were to be released from the point source during the duration of the chemotactic event. It is my guess that this is precisely what happened, given that male-female species have a higher incentive to produce a volatile cue (obligate mating) than

hermaphrodites (self-fertilizing). Let us now address the first condition of this assumption: an insufficient chemical gradient is present at the time of the assay. This could only be answered by the knowledge of how quickly the attracting compound moves through 1.5% agar during the amount of time that it would take to absorb into the agar (which is the deciding event for when males are placed on the assay). Upon recreation of this assay, it took approximately 20 minutes for a drop of water to absorb onto a 2 mm layer of 1.5% agar mounted on a glass microscope slide (as opposed to the several hour overnight incubation period used in the Simon and Sternberg (2002) study, perhaps explaining the discrepancy between these findings). Although we do not know how quickly the *C. elegans* attractant moves through agar, I have come to understand that the *C. elegans* pheromone has a significant lipid component, which would probably not facilitate effective distribution in a polar environment. The distinction between volatile and nonvolatile cues would be useful in clarifying these findings. This could be easily done if the paralyzing agent was removed and response to each scoring region was observed. It is therefore surprising that Chasnov et al. instead chose to create a third volatile assay (see Figure 3), which further verified that females did indeed produce a volatile cue.

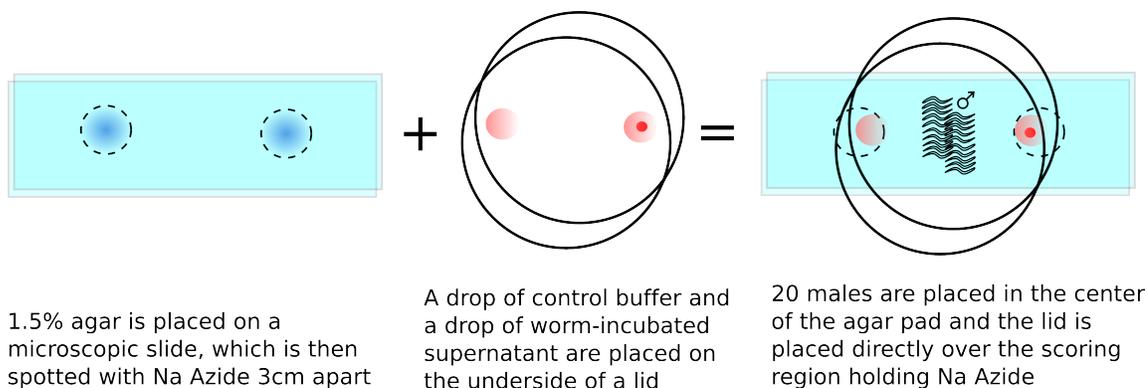


Figure 3: Chasnov et al. (2007) Assay used to score for male volatile chemoattraction to females[9]. (buffer = pink) (cue = red dot) (buffer + cue = pink + red dot) (sodium azide = dark blue)

From these findings, I conclude that *C. remanei* and *C. brenneri* females have a volatile cue. Whether or not hermaphrodites have a cue or females have a nonvolatile cue cannot be concluded from this experiment.

Lastly, Chasnov et al. addressed the source of the female volatile cue by performing a set of laser ablations on *C. remanei*. They ablated the somatic gonadal precursor cells, Z1 and Z4, and found that females did not produce an attractive cue, suggesting that the female volatile cue is made in (or depends on) the somatic gonad. Next they ablated the germ line gonadal precursors, Z2 and Z4, and found that females were still attractive, suggesting that the germ line is not necessary for production of a female cue^{**}. Lastly, they ablated the anchor cell, which allowed for full formation of the gonad but prevents the development of the vulva. They report that anchor cell-ablated females are still attractive, despite the lack of a vulva, suggesting that the release of the cue did not depend on the vulva. This would make sense, given that a volatile cue would likely diffuse through the cuticle.

Developing a new bioassay

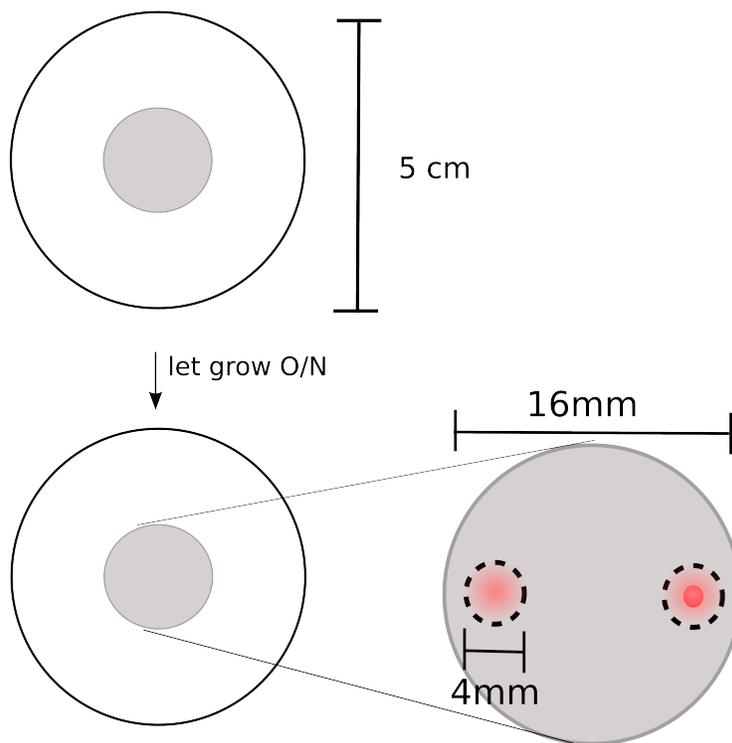
I have reproduced both the Simon and Sternberg (2002) and Chasnov et al. (2007) experiments. I do not favor the use of uncoordinated hermaphrodites, not only because I found them to be surprisingly coordinated, but also because the control for this experiment is the manual disruption of the bacterial lawn, with the assumption that making it look as unkempt as the area left behind by the uncoordinated hermaphrodites would suffice. I have observed that nematodes are able to burrow into the agar surface, sometimes leaving the

^{**} When Z2 and Z3 are ablated, the somatic gonad fails to extend its arms properly (which normally depends on germ line proliferation); thus an abridged somatic gonad forms as a result.

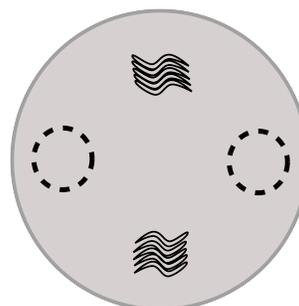
surface looking as if it were degraded via enzymatic secretion^{††}. This phenomenon has yet to be investigated, however I would not be surprised if the agar surface previously occupied by the hermaphrodite experienced changes different from the addition of a sex pheromone. This leads me to support the collection of a cue into a buffer, such that response in the buffered region may be compared to response in a cue-conditioned region. This approach provides two advantages: removal of plate-to-plate variation and removal of user bias. To address the first point, each agar plate has its own variations (moisture, texture, angle of surface, etc.) that could affect the results between plates. This variation leads me to support the observation of nematodes when presented with both the cue and control on the same agar plate. Lastly, I do not support the use of a paralyzing agent, given that I have no information about the chemical distribution of unidentified compounds and hence have no idea if a sufficient gradient would be present during the experiment. I began using several types of behavior assays and appreciated the absence of a paralyzing agent. Without a paralyzing agent, I may observe nematodes entering a region and responding (reverse, turn, re-enter). After much trial-and-error, I have developed an assay that reproducibly demonstrates the production of a *C. elegans* hermaphrodite sex pheromone (see Figure 4).

^{††} I am reminded of a popular clothing brand, Raquel Allegra, which sells extra soft t-shirts. She reveals that her secret to the soft textile is that they are recycled from old shirts worn by prisoners at the LA County Jail, whose sweat simply degrades the cotton fibers.

place 3 drops of E. coli OP50 on an agar plate



let the drops settle in (takes approx 5 seconds)
 0.6 μ L control buffer on one side, 0.6 μ L cue on other
 place 10 males down (at the same time) at two points equidistant from the foci of the scoring regions



record for 20 minutes

Figure 4: Because our behavior assay does not utilize a paralyzing agent, recording is required to observe individuals as they move in and out of scoring regions. (buffer = pink) (buffer + cue = pink + red dot)

parameter	rationale
16mm bacterial lawn	I chose a magnification such that I could capture enough detail of the worm to gather sufficient information about the edge of the worm along the boundaries of the conditioned region. This allowed for approximately 18mm diameter in the camera's field, thus establishing the reason for a 16mm bacterial lawn.
4mm scoring region	A 0.6uL drop of buffer spread to a diameter of approximately 4mm, thus I set my scoring region to be 4mm.
20min assay	An assay that was less than 10 minutes did not allow worms to sufficiently sample the entire lawn when control was placed on both sides. An assay that was greater than 30 minutes allowed for males to become adapted to the cue and their response began to change. Also after about 25 minutes, the borders of the cue region were less defined as males began to track cue out of the scoring region.
6hr worm secretion collection, at 1 worm per microliter	I began with 6hrs as Chasnov et. al (2007) described, and used a concentration of 1 worm per microliter. This concentration was suggested to me by Ryan Baugh, after he drew his own conclusions about density-dependent stress in liquid culture and advised that 1 worm per microliter seemed unstressful. I saw that individuals were thrashing in a healthy manner after 6 hours and only began to show signs of fatigue after 10 hours.

Figure 5: Rationale for chosen parameters of the behavior assay

I describe the rationale for every parameter in Figure 5, with the goal of collecting high concentrations of hermaphrodite-derived cue (without causing stress) and eliciting a reproducible male response to their attractant. After placing a drop of buffer and hermaphrodite-incubated buffer on the bacterial lawn, I would immediately place 5 males on each dropping area equidistant from the foci of the scoring region (see Figure 4). The immediacy of the drop absorption on a bacterial lawn (5 seconds) is a great advantage over drop placement onto agar (ranges from 2-20 minutes depending on the thickness and %

agar). The first observation that I made was that the males would distribute randomly (star-shaped dispersion) and move quickly throughout the lawn until they came to the region conditioned with the hermaphrodite-derived supernatant. The males would then slow down and reverse such that they would stay within that area (see Figure 6). Once other males would enter this region, they would repeat the same behavior and often times attempt to mate with other males.

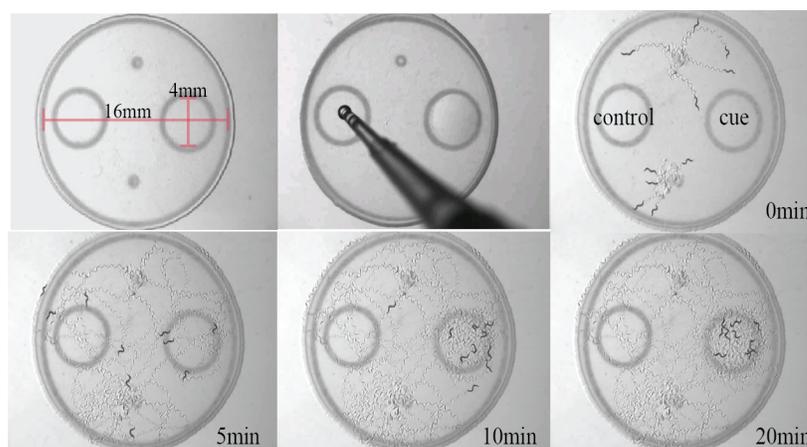


Figure 6: Observation of male attraction to a hermaphrodite-incubated buffer (right circle)

The next step would be to establish a metric for scoring attraction to the cue. I can appreciate why the sodium azide method is so popular, given that you can start the assay and come back an hour later to score by simply counting the # males paralyzed in either region. I could see by the star-shaped distribution of their dispersal that they would distribute evenly upon release (see Figure 6) therefore scoring with an azide technique would hide any holding effect of a cue. I could do one of two things: 1) adopt the azide technique and allow the cue to settle in overnight with the hope that a chemical gradient would form and the cue would not disintegrate during this time or 2) figure out how to score worm attraction without a paralyzing agent. I favor the second method, not only

because the first method relies on hidden information, but also because I find it informative to watch males interact with the cue time and time again. I could see that they demonstrate several types of taxis: their heads move side to side (klinotaxis) and reverse when their head region, but not tail, became removed from the conditioned region (tropotaxis). I also observed kinesis, as males tended to speed up once they left the conditioned region and slowed down upon re-entering. I began to score “worm events” as the duration of time between when each worm entered a scoring region and when it left the scoring region (see Figure 7).

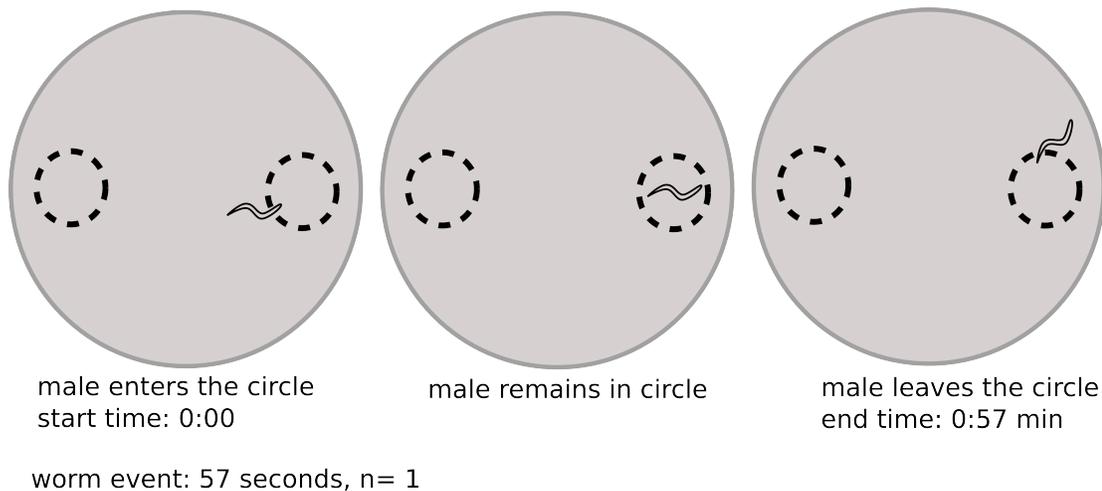
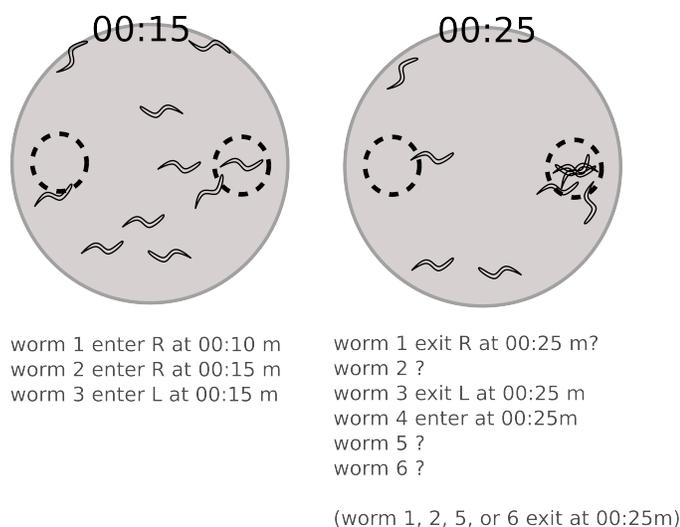


Figure 7: Calculating worm events: start time when the worm enters the circle, end time when the worm leaves the circle

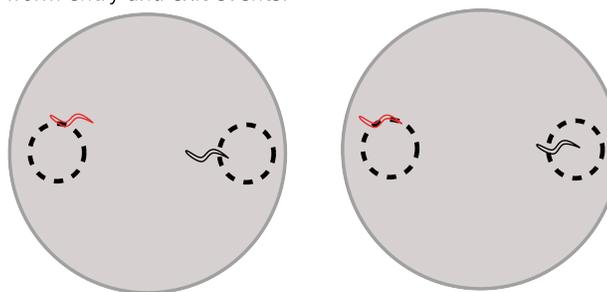
It can become challenging to keep track of 10 worms that are entering the circle, and it is also very time consuming to manually score worm events. Although I still like the approach of comparing time spent in each scoring region, I dislike the metric of scoring worm events for several reasons (see Figure 8). Perhaps the most obvious problem is when the experimenter must manually score entry and exit times when there are ten worms simultaneously moving throughout both regions. When worms cross over, the experimenter

must make a best guess to assign worm identity. This becomes particularly challenging when multiple worms collide, making the decision arbitrary. There are also many gray zones, for example when a worm almost entirely leaves the scoring region but reverses back. An experimenter must decide whether to score that as one worm event or two. Trials can be performed one worm per plate but that would be very time consuming.

1. When worms crawl over each other, it is difficult to maintain worm identity and thus it is difficult to say when the start and end time is for individual worms.



2. User bias: aside from using a best-guess for worm identifies during overlapping events, there is also user bias in defining worm entry and exit events.



Is the **red worm** skimming the scoring region? How is that scored?
Did the **black worm** leave and re-enter if they reversed with their tail still in the scoring area?

Figure 8: Problems with user error/bias with calculating worm events

Lastly, the metric of scoring each worm event as $n=1$ can be problematic. With a trial of 20 minutes, there may be hundreds of worm events and thus n can be > 100 . If one worm enters the scoring region repeatedly, each reentry will increase the value of n , therefore increasing the significance (and decreasing the standard deviation) of that phenotype with false amplification.

I was given the suggestion to use an automated approach to scoring attraction, such that the labor and bias of scoring manually would be alleviated. Dmitriy Kogan wrote a program for this purpose, using a set of optical filters to exaggerate the contrast between the worms and the background, which would allow for the detection of worm pixels within each scoring region (see Figure 9).

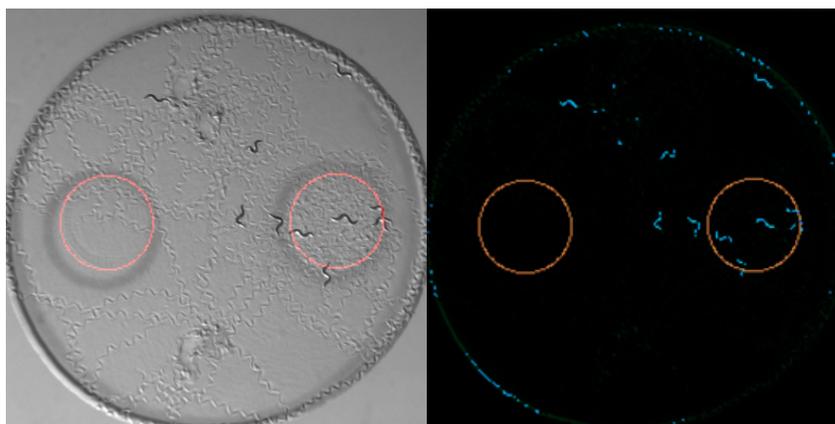


Figure 9: Optical thresholding allows for the detection of worm pixels in each scoring region, which could then be used for comparison

The extraction rate is one frame per second (total 1200 frames per 20 minute trial), which allows for sufficient resolution of worm entry and exit.

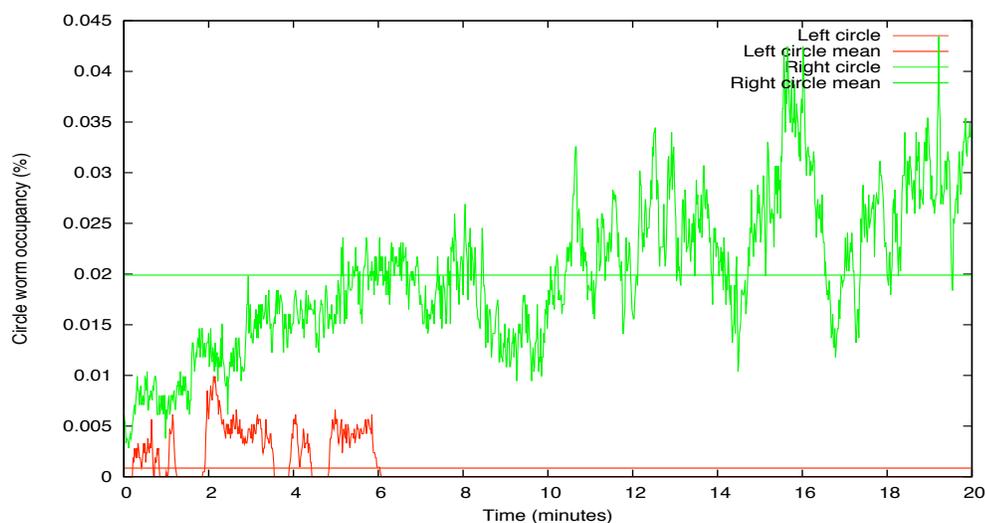


Figure 10: Data output in the form of percentage worm occupancy in each scoring region, over 20 minutes (1 frame/second)

The output shows the percentage worm occupancy, per scoring region, over 1200 frames (see Figure 10). If worms are attracted to a region and accumulate, this percentage worm occupancy increases over time (see Figure 10, green line). If the worms are not attracted to a region, this percentage would rise with entry, plateau during their duration in the scoring region, and decline with exit (see Figure 10, red line). To be certain that this method finds individual worms accurately, I took a segment of video where two worms in a scoring region exit in sequence (see Figure 11).

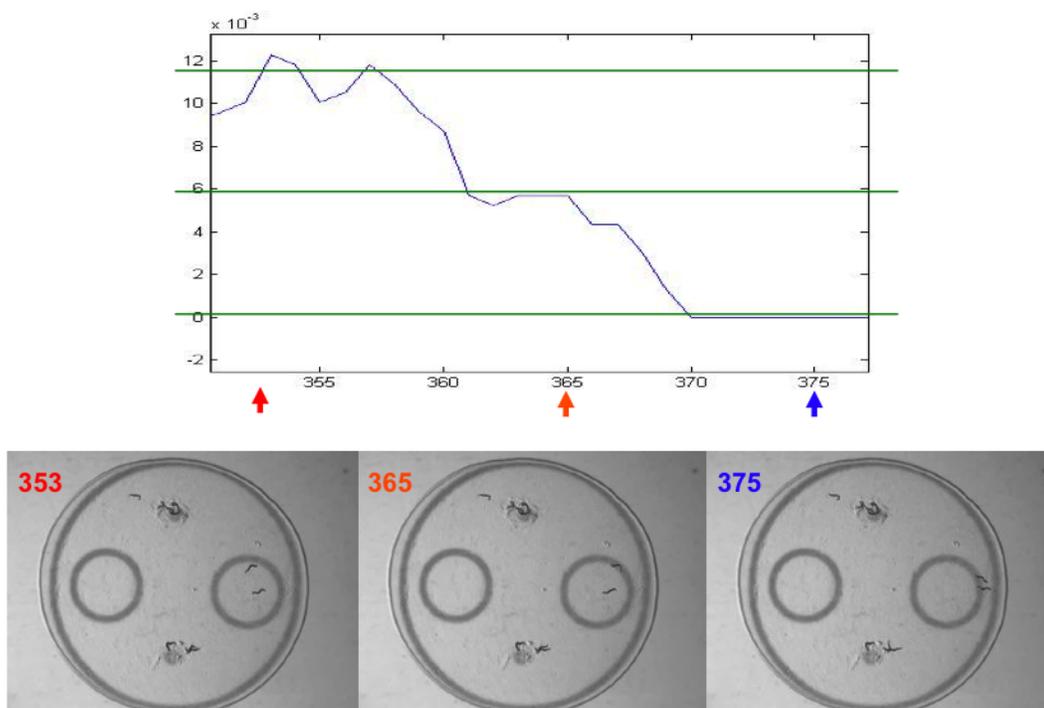


Figure 11: The data reflects changes in worm occupancy over time

The data output reflects these events accurately, diminishing in equal ratios of worm occupancy upon exit.

I then take the integral under each curve and apply them to an attraction index:

$$\frac{\% \text{ W.O. cue} - \% \text{ W.O. control}}{\% \text{ W.O. cue} + \% \text{ W.O. control}}$$

Time spent equally in each region would result in a score of 0, perfect attraction to the cue is +1, and perfect repulsion to the cue is -1.

In my first set of experiments, I incubate 50 *C. elegans* L4 hermaphrodites (the life stage preceding sexual development), 50 young adult (YA) hermaphrodites (the life stage where the vulva first becomes exposed to the external environment), 50 adult hermaphrodites (the life stage where eggs develop), and 50 adult males each in 50 μL M9 buffer. They are

incubated at 20°C for 6 hours and the supernatant was collected and stored at -20°C until the time of the experiment. I report that *C. elegans* males are attracted to young adult and adult hermaphrodites, but not to L4 hermaphrodites or adult males (see Figure 11).

***C. elegans* males to hermaphrodites at different developmental stages and males**

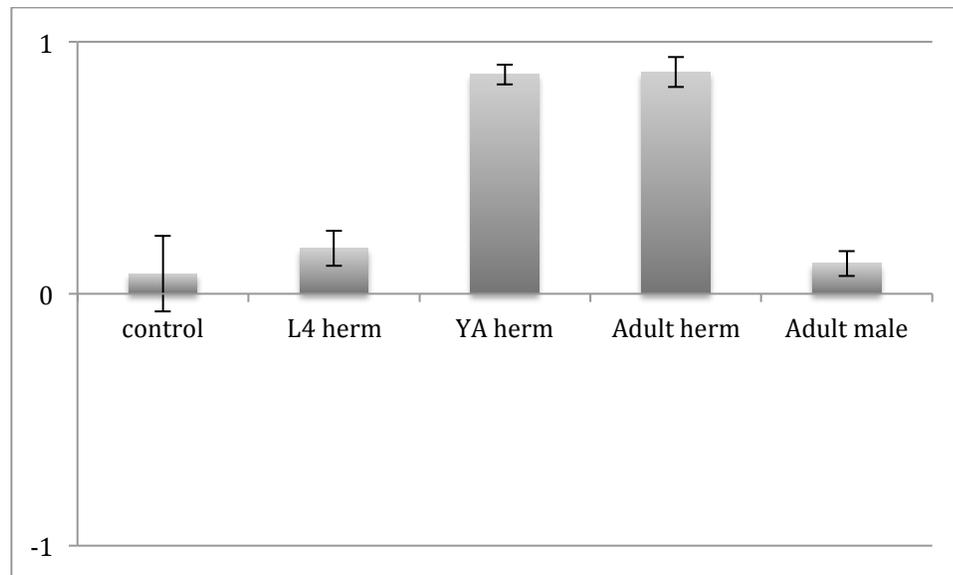


Figure 11: *C. elegans* males are attracted to young adult and adult hermaphrodites, but not to L4 hermaphrodites or adult males. -1= perfect repulsion, +1= perfect attraction, 0=no difference

These results support the findings from the Simon and Sternberg (2002) study and conclude that *C. elegans* indeed produces a sexually dimorphic cue. It makes sense that L4 hermaphrodites are not attractive, given that they are yet unable to mate. The young adults and hermaphrodites are equally attractive, which seems more advantageous for the young adult since they have not yet produced eggs, however the adults may continue to produce more eggs and would likely still benefit from sexual reproduction.

Next I looked to see if closely related species followed the same rules of attraction. I tested several species from the genus *Caenorhabditis*: the hermaphroditic species *C. briggsae* and

the male-female species *C. remanei*, *C. brenneri*, and *C. sp5* (see Figure 12).

Conspecific attraction of males to *Caenorhabditis* females or hermaphrodites

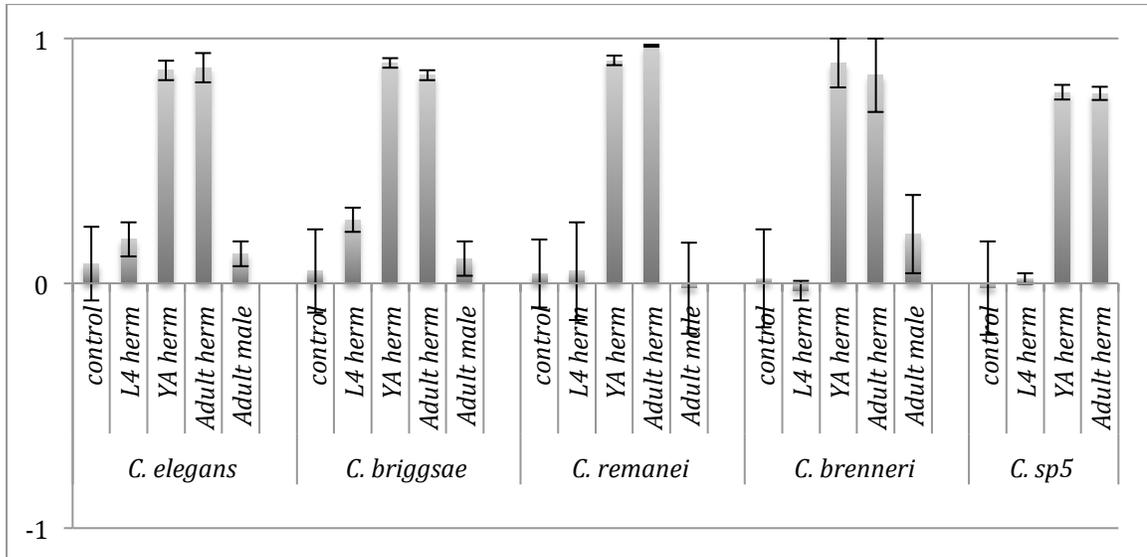
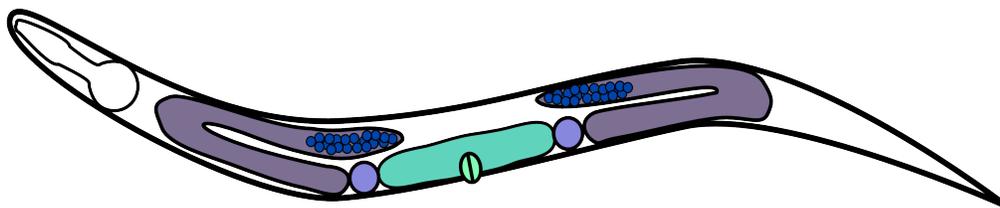


Figure 12: Conspecific chemoattraction of males from the species *C. elegans*, *C. briggsae*, *C. remanei*, *C. brenneri*, and *C. sp5* to females or hermaphrodites

I found that *C. briggsae*, *C. remanei*, *C. brenneri*, and *C. sp5* females and hermaphrodites also become attractive following the L4/adult molt. Male-incubated buffer did not attract conspecific males (*C. sp5* not tested), thus demonstrating that several species from the genus *Caenorhabditis* have sexual dimorphic cues.

Next I aimed to identify the source of the cue and began with gonad ablations. Because the mate finding cue is sexually dimorphic, the gender-specific reproductive organs are a good starting candidate.



Reproductive system part	Adult Tissue	Precursor/cell present at hatching
Germ line 	mitotic and meiotic germ cell oocytes sperm	Z2, Z3
Somatic gonad 	distal tip cell gonadal sheath spermatheca sp-ut valve uterus anchor cell	Z1, Z4
Egg-laying apparatus 	vulva VC neurons ventral hyp	P3-8
	HSNR/L neurons ABp(l/r)appappa	HSNL/R
	vulval muscles uterine muscles	M

Figure 13: Precursor cells to the three main components of the *C. elegans* hermaphrodite reproductive organ. Adapted from Lints, R. and Hall, D.H. (2010) *WormAtlas*[11].

The precursor cells for the three main components of the reproductive organ (germ line, somatic gonad, and egg-laying apparatus) are very well characterized in *C. elegans* [11], making cell-specific laser ablations an ideal method for removing potential sources of the cue. Laser ablation of Z1, Z2, Z3, and Z4 during the L2 larval stage give rise to a hermaphrodite which entirely lacks a reproductive organ, including the vulva, germ line, and somatic gonad. I performed ablations of Z1, Z2, Z3, and Z4 at the L2 larval stage and

checked for lack of the gonad (via Nomarski optics) at adulthood before incubating 20 surgically altered hermaphrodites in 20 μ L buffer for 6 hours. I used this worm-incubated buffer to test males on the same assay described above (see Figures 6, 10 and 11). Males are not attracted to gonadless hermaphrodites (see Figure 14), suggesting that the vulva or gonad is required for the production and/or secretion of the mate finding cue.

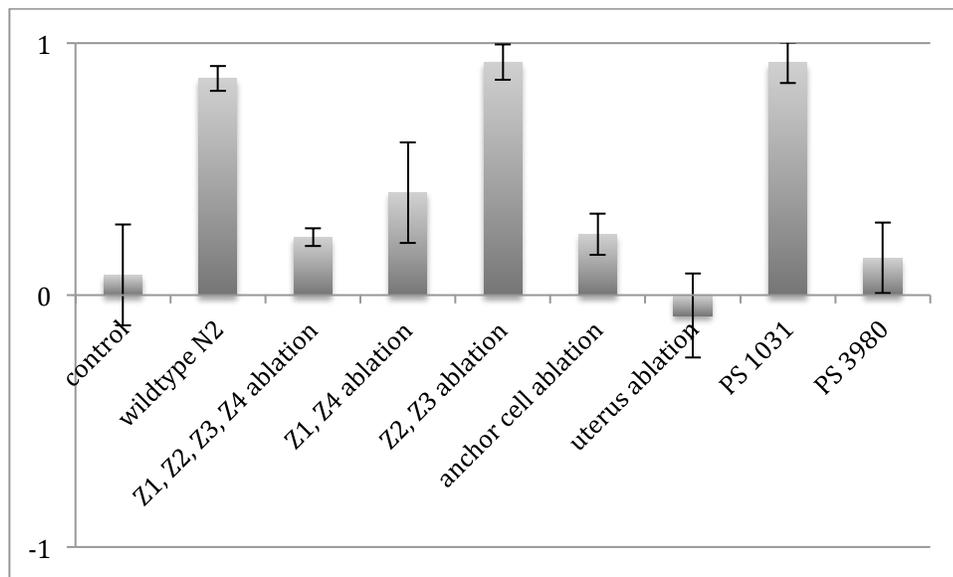


Figure 14: *C. elegans* males are attracted to hermaphrodites lacking germ cells, but not attracted to those lacking the somatic gonad. *C. elegans* males are attracted to the vulvaless mutant, PS1031, but not to the vulvaless mutant PS3980, anchor cell ablated, or uterus precursor ablated hermaphrodites

Next I performed Z1 and Z4 ablations, which allows for development (but not proliferation) of the germ line precursors and found that males are not significantly attracted to these hermaphrodites, which suggests that the vulva or somatic gonad are important for the production and/or secretion of the attractant. Z2 and Z3 ablated hermaphrodites develop a vulva and an abbreviated somatic gonad but lack a germ line. These hermaphrodites significantly attract males, thus suggesting that the germ line does

not play a role in the cue production. Ablation of [Z1, Z2, Z3, Z4] or [Z1, Z4] both prohibited the development of a vulva, therefore it is still not clear whether the vulva is a necessary source or outlet for the attractant. It would not be useful to perform an ablation of Z1 or Z4 alone, because half a gonad would still form along with the vulva, thus not answering the question of whether the vulva is simply an outlet for gonad-produced cue or if the vulva produces a cue. Cellular ablation of the vulva is not as straightforward as gonad ablations, but can be accomplished with the ablation of the anchor cell, a single cell of the somatic gonad that sends signals to epidermal precursors to become vulval cells (see Figure 14)[12].

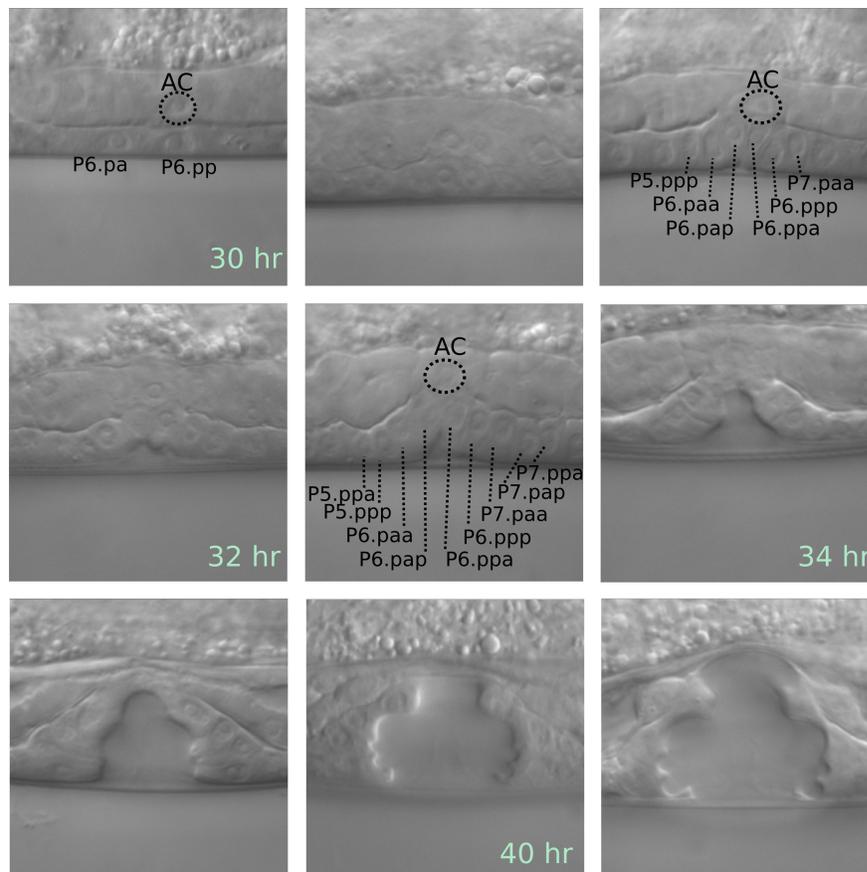


Figure 14: The anchor cell (AC) induces several posterior daughters of the Pn ventral neuro-ectoblasts (P5.p, P6.p, P7.p) to become vulval cells. Induction begins around 30 hours posthatch

The anchor cell is necessary and sufficient for vulval development, through the expression of epidermal growth factor (EGF)-like ligand LIN-3 [12]. Ablation of the anchor cell must be performed prior to L2/L3 molt (see Figure 14, 30 hours posthatch), as it will have already induced the vulval precursor cells (VPCs) to begin adopting their 1° or 2° fates [13]. The anchor cell comes from one of two possible daughters of the somatic gonad cell Z1: (Z1.ppp) or Z4 (Z4.aaa) [14]. A challenge exists due to the fact that too-early ablation of the anchor cell will induce the other daughter to take over the fate of the anchor cell, therefore the ablation must be performed shortly before the L2/L3 molt^{‡‡}. Because the anchor cell induces the VPCs through the expression of LIN-3, an alternative approach would be to test *lin-3* hermaphrodite mutants. Because orthogonal approaches will best illuminate the most likely answer, I have tried all of the aforementioned methods. Ablations of the anchor cell directly preceding the L2/L3 molt caused hermaphrodites to develop a fully formed gonad without a vulva, as verified by Nomarski optics. These vulvaless hermaphrodites are not attractive to the males (see Figure 13), which suggests that the vulva is required for the production or release of the male attractant, contradicting the finding of Simon and Sternberg (2002). Next I tested the response of males to hermaphrodite-incubated buffer using the same vulvaless mutant in the Simon and Sternberg (2002) study: PS3980 (*(let-23(sy1) unc-52(e444); dpy-20(e1282) lin-3(n378); him-5 (e1490)*). These PS3980 vulvaless mutants do not significantly attract males, confirming the finding from Simon and Sternberg (2002). Next I tested a different

‡‡ My advice to anyone attempting to identify the anchor cell preceding the L2/L3 molt is to look for the “evil” looking cell. I cannot explain why this works, it just does. Perhaps the nuclear shape is slightly more irregular, casting slightly more sinister shadows upon illumination.

vulvaless mutant strain: PS1031 (+ *let-312(s1234) lin-3(n378) + unc-22(s7)/ unc-24(e138) + lin-3(n1059) dpy-20 (e1282)*) and found that these vulvaless hermaphrodites significantly attract males. I am somewhat skeptical about genetic mutants, given that we do not understand the full activity profile of *lin-3*. There is an explanation for why one vulvaless mutant attracted males and the other did not, however we do not have access to this answer without further investigation of the methods used for production of these mutant strains and perhaps a deeper and broader understanding of *lin-3* activity. In this circumstance, I favor the cell ablation for the simple rationale that I could see that the anchor cell is eliminated prior to the L3 molt and that the VPCs adopt a 3° fate, whereas the anchor cell is still present in the vulvaless mutants and could perhaps induce a partial non-tertiary fate that is otherwise undetectable by Nomarski optics.

Next I performed ablations of the dorsal and ventral uterine precursors, such that the vulva still develops (dorsal uterine precursors: Z1.pap, Z4.apa; ventral uterine precursors: Z1.ppa, Z4.aaa, Z4.aap; anchor cell precursor Z1.ppp left intact). As a result, the spermathecal chamber were also removed. These hermaphrodites did not elicit attraction, therefore I conclude that the mate-finding cue is produced by the spermathecal chamber or uterus and requires the vulva for release into the outer environment.

Lastly, I performed Z1-Z4 ablations in females or hermaphrodites of other *Caenorhabditis* species to see if secretion of the cue requires the presence of the reproductive organ (see Figure 15).

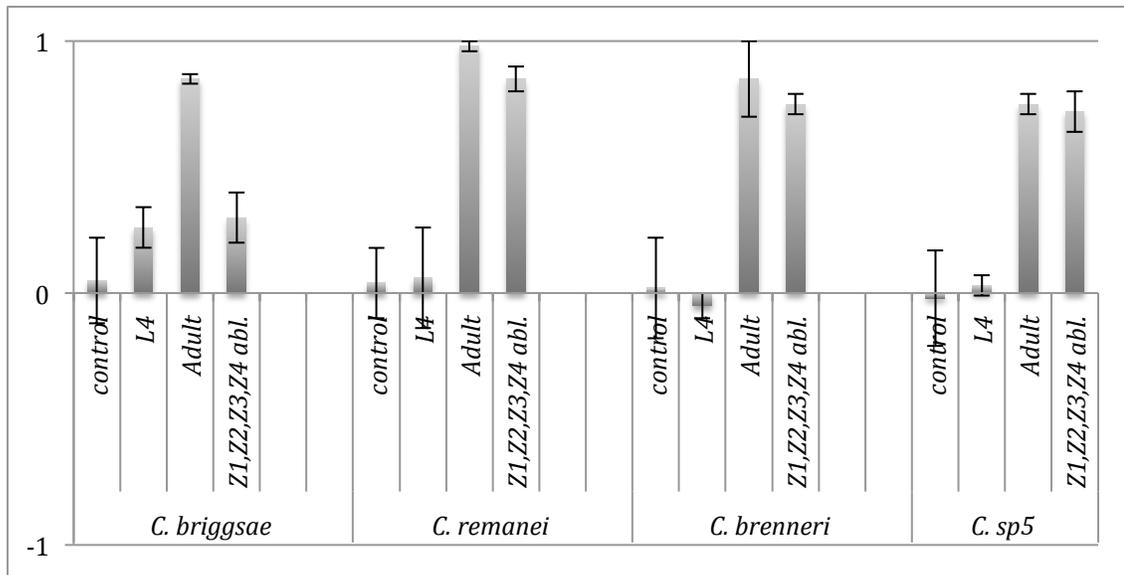


Figure 15: The hermaphroditic *C. briggsae* requires the reproductive organ for production or release of the male attractant, whereas the male-female species *C. remanei*, *C. brenneri*, and *C. sp5* do not.

I report that *C. briggsae* requires the hermaphrodite gonad to produce a male attractant, however *C. remanei*, *C. brenneri*, and *C. sp5* do not require the female gonad to produce a male attractant. This is interesting because the divide separates hermaphroditic species from male-female species. Perhaps male-female species produce a volatile cue, as concluded by Chasnov et al. (2007), and may be produced by an organ other than the gonad, escaping through the mouth, anus, excretory pore, or cuticular diffusion.

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CHAPTER 3

Investigation of aqueous and volatile pheromones in the genus *Caenorhabditis*

Introduction

In the soil, *C. elegans* lives at the air-water interface and is exposed to a wide variety of both aqueous and volatile chemicals. Many chemical attractants and repellants for *C. elegans* have been identified through a large-scale screen that include both volatile and non-volatile components. Bargmann and Mori (1997) suggest that volatile molecules may be used by *C. elegans* for long-range chemotaxis, whereas water-soluble molecules are used mainly for short-range chemotaxis [1]. Bargmann, Hartweg, and Horvitz (1993) report that chemotaxis to volatile odorants require different sensory neurons than those that detect water-soluble attractants [2].

White et al. (2007) identified specific neurons responsible for chemotaxis towards the mate-finding cue, demonstrating that *C. elegans* males chemotax to a hermaphrodite-derived source via the TRPV (transient receptor potential vanilloid) channel encoded by the *osm-9*, *ocr-1*, and *ocr-2* genes. OSM-9 is necessary in three classes of sensory neurons: the AWA and AWC olfactory neurons and the male-specific CEM (cephalic companion) neurons. Ablation of these sensory cells following the 4th larval molt lead to impaired attraction, whereas ablations performed before sexual maturation leads to unimpaired attraction, presumably through adoption of cell fate by a related precursor. However, this study does not separate volatile and nonvolatile components of the mate-finding cue, leaving much to elucidated about the nature of the mate-finding cue.

Results

The evidence reported by White et al. (2007) suggests that *C. elegans* hermaphrodites produce and secrete a volatile male attractant, given that the volatile sensory cells, AWA and AWC, are both necessary for the successful chemotaxis of males to the point source [3]. Here I describe the White et al. (2007) method of cue collection (see Figure 1) and metric for scoring male chemoattraction to a hermaphrodite-derive cue (see Figure 2).

Grow synchronized L1 N2 at 10worm/ μ L for 3 days at 25°C with 1% v/v HB101 on the first day and 1% each day thereafter

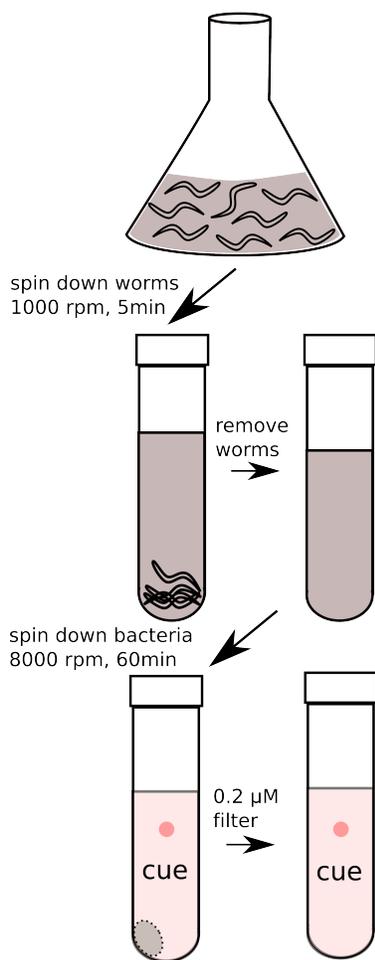


Figure 1: Method of N2 hermaphrodite cue collection, as described by White et al. (2007) [3].

20 μ L of the control and cue are dropped on opposite sides of a 5cm plate and allowed to dry for 1 hr, after which 240 μ L of 2% v/v HB101 is spread and allowed to dry for 30 min.

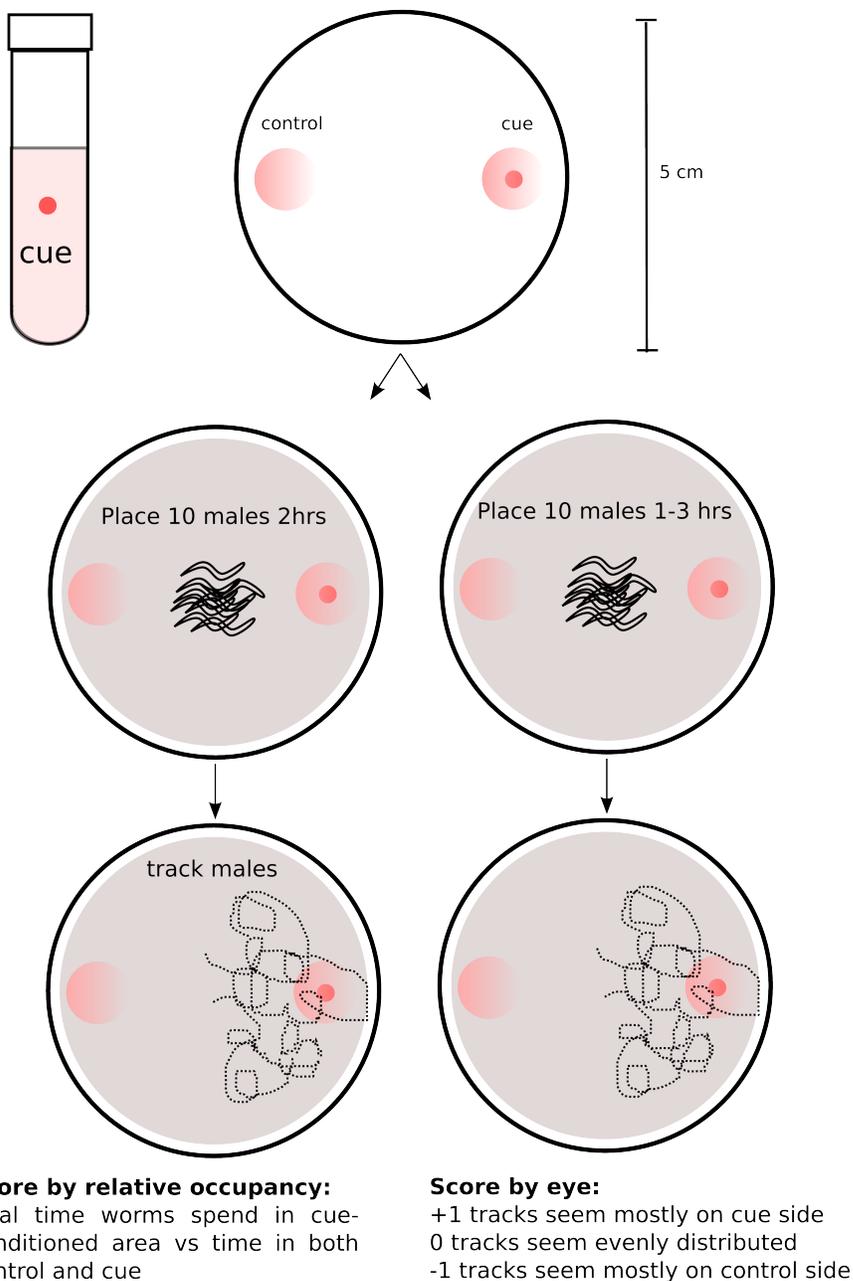


Figure 2: Method of scoring male chemotaxis to cue, as described by White et al. (2007) [3]. Control (S. complete buffer) = pink, Cue in buffer = red dot in pink, bacteria = gray.

The method described by White et al. (2007) takes worms and bacterial supernatant and

filters both through centrifugation and filtration (see Figure 1). A drop of the filtered supernatant is placed on one side of a 5cm agar plate and a drop of *S. complete* is placed on the opposite side. I disapprove of the use of *S. complete* as the control in these studies, given that *E. coli* produces diffusible attractants [4]. The appropriate control for this experiment should at the very least include incubated and filtered *E. coli* HB101. For this reason, I cannot be certain that these experiments were simply attraction to food, especially since HB101 is typically grown in Terrific Broth, which contains additional extract from the yeast *Saccaromyces cerevesiae*. The other point that I would like to address is that hermaphrodites are not found to be significantly attracted to the cue in the White et al. study, thus lending credibility to the conclusion that the cue is indeed a mate-finding cue. However, I have come to find through my own observations that hermaphrodites do not respond as robustly to dilute odors as males, perhaps attributable to the faster speed of males and thus ability to chemotax more effectively than hermaphrodites in a given allotment of time. For these reasons, I have decided to investigate the *C. elegans* hermaphroditic volatile cue using the following control (see Figure 3):

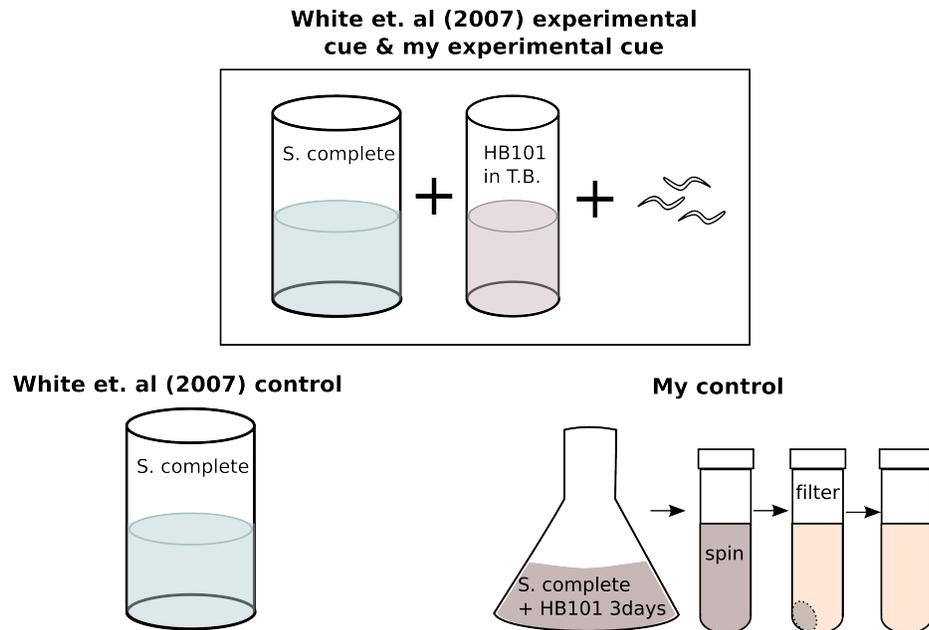


Figure 2: Control used in the White et al. (2007) study[3] and the control for my volatile assay

This control will clarify attraction to food with cue vs. food alone. Because we do not know anything about the *S. complete*-incubated *E. coli* HB101 metabolites, the inclusion of these elements are essential to elucidate a possible hermaphrodite-derived volatile attractant. To investigate this matter, I have tested hermaphrodites in *S. basal* alone and did not find evidence of a volatile attractant. Perhaps the presence of food is necessary, given the evidence that other developmental and behavioral processes require sufficient nutrition. I use the same experimental method of collecting cue, however modified a few parameters of the White et al. (2007) assay:

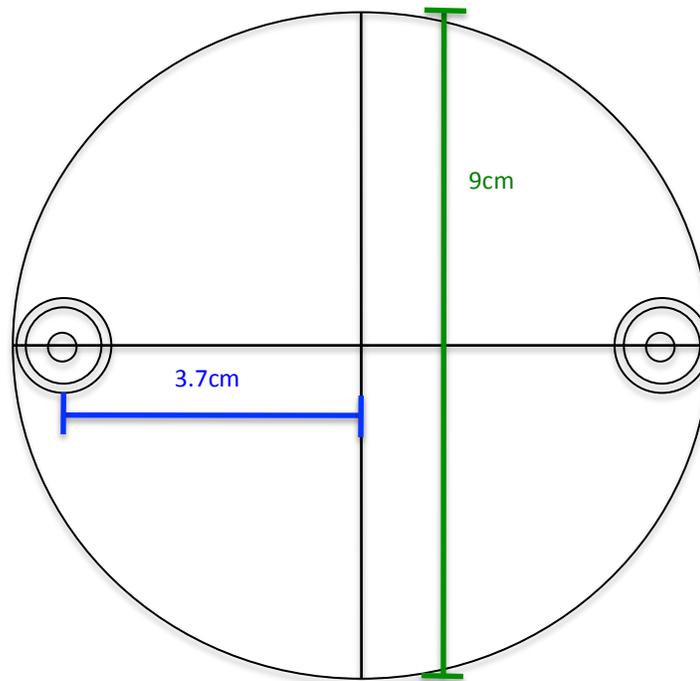


Figure 3: Experimental parameters of my volatile assay.

1) 9cm agar plate with no food (instead of 5cm plate with food): Because volatile cues are thought to play a role in long-range attraction, I expanded the diameter of the assay to see if males could chemotax to the point source from a distance $> 30x$ their length. I also eliminated the presence of food on the plate, such that my control would effectively clarify the attraction to food-derived cues only from the experimental cue or control (see Figure 3).

2) Cue plated on the lid (instead of on the agar plate): Because these experiments are designed to clarify a possible volatile cue, I placed the cue on the lid to test for volatile-only chemotaxis.

3) Sodium azide below the point source of cue and control: Paralyzing agents are useful when studying volatile chemoattraction, because individuals become paralyzed near the location that they are first attracted to. Even if males become adapted to the volatile

attractant, this method would show that they successfully located the point source before exploring other options.

After placing a drop of the hermaphrodite and food-derived cue and a drop of the food-derived cue under the lid, I place 10 males at the center of the 9cm plate and wait 2 hours before scoring for paralyzed males under either scoring region, using the following attraction index:

$$\frac{\#worms \text{ under cue} - \#worms \text{ under control}}{\#worms \text{ under cue} + \#worms \text{ under control}}$$

+1= perfect attraction to the hermaphrodite attractant over the food cue

0= equal attraction to the volatile attractant and food-derived attractant

-1= perfect attraction to the food-derived attractant over the hermaphrodite cue

To discriminate between attraction to volatile vs. nonvolatile attraction, I use separate assays: I place hermaphrodite-conditioned media on the lid above a 10-cm agar plate and place a drop of lyophilized hermaphrodite-conditioned media on a 5cm plate (as described in Chapter 2), respectively.

C. elegans males and hermaphrodites to the volatile cue

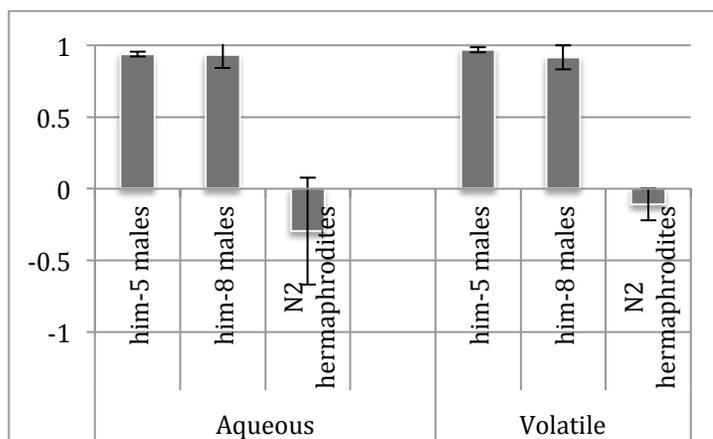


Figure 4: *C. elegans* males, but not hermaphrodites, are attracted to the volatile cue

I report that *C. elegans* males (taken from CB1490 *him-5(e1490)* and CB1489 *him-8(e1489)*) but not hermaphrodites, are attracted to the volatile cue over the food-derived cue (see Figure 4).

The Volatile Cue is Detected through the Sensory Neuron AWA

The ciliated neurons AWA and AWC are essential for chemotaxis to volatile odorants [2]. They are distinct from most of the chemosensory neurons in the amphid, both in shape and exposure to the environment. Their complex, wing-like shape are characteristic of olfactory neuron cell types in other animals [5] and they are protected by the amphid sheath thus allowing exclusive contact with volatile compounds capable of penetrating the amphid sheath [2]. To determine whether these sensory neurons play a role in chemoattraction to the volatile mating cue, I have performed laser ablation of AWA or AWC on male L2 larvae, allowing them to recover and develop into adult males before testing. I have shown that male attraction to the volatile cue is unaffected by loss of AWC, however is severely affected by the loss of AWA (see Figure 5). Both AWA and AWC ablated males exhibit wild-type attraction to the aqueous cue. These findings are reciprocated by analysis of mutants with genetic defects uniquely affecting AWA or AWC (see following section), suggesting that the volatile mating cue is detected through AWA.

Laser-ablated *him-5* males to the volatile cue

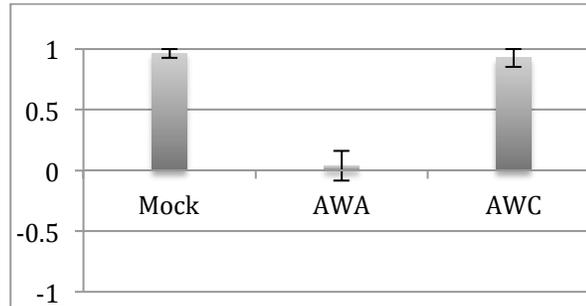


Figure 5: AWC-ablated *C. elegans* males, but not AWA-ablated males, are able to find the volatile cue

Chemotaxis towards the Volatile Cue Requires the TRPV Channel

Although AWA and AWC share in their ability to detect volatile chemicals, they have very different pathways and molecules required for signal transduction (see Figures 6 and 7). AWA recognizes odorants using *osm-9* encoded TRPV (transient receptor potential vanilloid) channels whereas AWC utilizes *tax-2* and *tax-4* encoded cyclic nucleotide-gated channels[6]. *osm-9* mutant males (CX10 *osm-9* (ky10), EG4172 *osm-9* (n1601);*him-5*, EG4173 *osm-9* (n1603);*him-5*) are defective in attraction to the volatile cue but exhibit wild-type attraction to the aqueous cue (see Figure 8 and 9), which is consistent with the finding that AWA-ablated males are defective in attraction to the volatile cue but not to the aqueous cue. *tax-2* and *tax-4* mutant males exhibit wild-type attraction to both the volatile and aqueous cue (see Figure 8 and 9), which is consistent with the finding that AWC-ablated males exhibit wild-type attraction to both components of the mating cue.

The TRPV channel in AWA further involves two other TRPV genes, *ocr-1* and *ocr-2* (*osm-9*/capsaicin receptor related)[7]. *ocr-1* and *ocr-2* single mutants exhibit no defect on their own, however *ocr-1*;*ocr-2* double mutants exhibit significant defect in attraction to

both the volatile and aqueous cue (see Figure 8 and 9). This overlaps with the findings of White et al. (2007), whom also found that the *osm-9* single mutants and *ocr-1;ocr-2* double mutants exhibit defect to the mating cue whereas the individual single mutants exhibit wild-type attraction, concluding that OCR-1 and OCR-2 are most likely redundant with one another[3]. The assay used in the White et al., 2007 study most likely contained combined aqueous and volatile components of the cue, explaining consensus over some results and deviation in others. These components have been separated in an effort to clarify the role of specific amphid neuron cells and signaling molecules to specific components of the mating cue. To that end, I have found that *osm-9* is only required for the volatile, but not the aqueous cue, whereas *ocr-1* or *ocr-2* is required for both the volatile and aqueous cue.

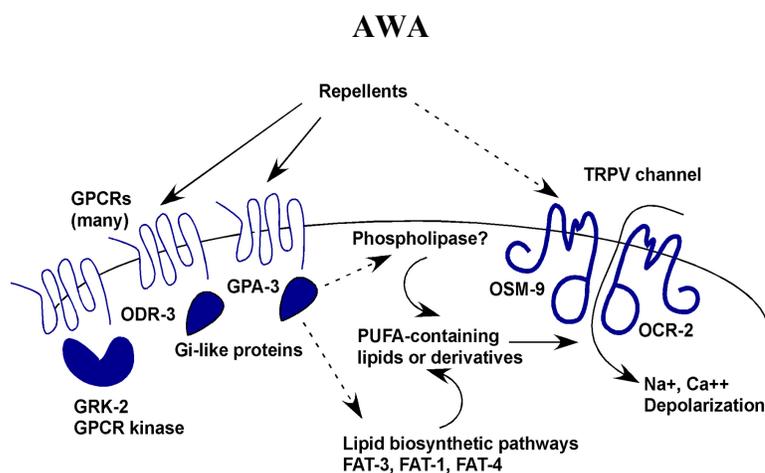


Figure 6: Potential signal transduction pathway for odor detection in the AWA and ASH cilia. Taken from Bargmann, C.I. (2006) *WormBook*[8].

Chemotaxis towards the Volatile Cue is Mediated through the G α Protein ODR-3 and the Nuclear Hormone Receptor ODR-7

The G α protein ODR-3 is strongly implicated in the function of *C. elegans* olfaction, most likely activating the OSM-9, OCR-1/OCR-2 containing TRPV channel in AWA. *odr-3* mutant males fail to respond to the volatile mating cue, but is attracted to the aqueous cue (see Figures 8 and 9), implicating ODR-3 in the detection of the volatile cue, most probably through AWA. Although the expression of *odr-3* and ODR-3 is more abundant in AWC than in AWA and that *odr-3* mutation creates more severe visible defects in AWC, *odr-3* is more critical for AWA function[9]. This continues to support the ablation results previously stated.

Because *odr-3* might act directly through the AWA-specific G-protein coupled receptor, ODR-10 [9], *odr-10* mutant males were also tested. *odr-10* mutant males display strong attraction to both the volatile and aqueous mating cue (see Figures 8 and 9), leading to the conclusion that *odr-10* probably does not play a role in detection of the mating cue.

The *odr-7* gene encodes an olfactory-specific member of the nuclear receptor superfamily and is required for the function of AWA[10]. It is expressed predominantly in the AWA neurons, throughout all postembryonic stages, most likely regulating transcription and perhaps playing a role in chemotaxis behaviors[10]. *odr-7* mutant males fail to chemotax towards the volatile cue, but respond to the aqueous cue (see Figure 8 and 9), implicating it in the detection of or response to the volatile cue.

Chemotaxis towards the Volatile Mating Cue is Faster in *tax-2* and *tax-4* Mutants

Observation of *tax-2* and *tax-4* mutants' response to the volatile mating cue has revealed that the mutant males actually respond faster to the volatile mating cue (see Figure 10). It seems that *tax-2* and *tax-4* mutants pirouette less and deviate less from the trajectory towards the volatile cue.

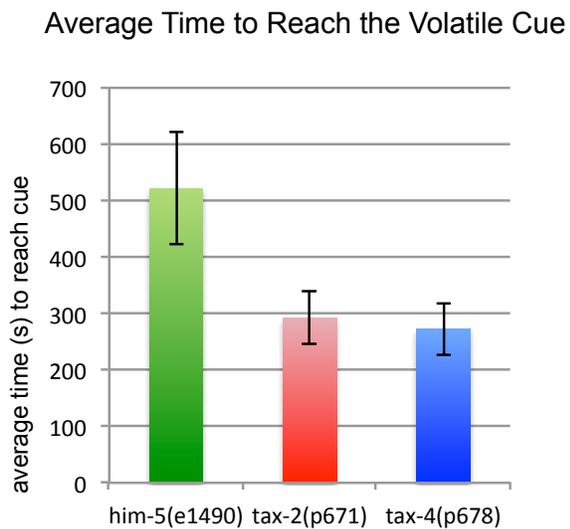
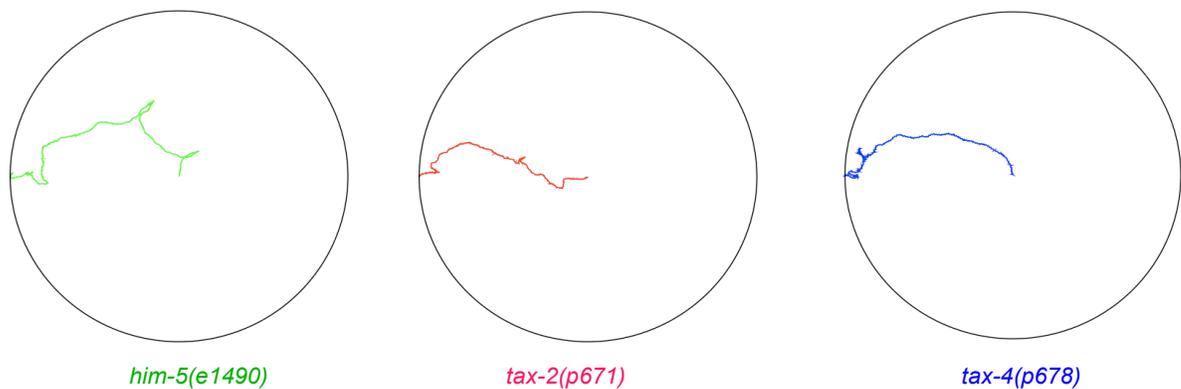


Figure 10: Chemotaxis to volatile cue is faster in *tax-2* and *tax-4* mutants. Start= center, cue=left.

Before making assumptions about differences in *tax-2* and *tax-4*-mediated signal transduction, I would have to rule out a possible difference in velocity. Because my volatile chemotaxis assay tests 10 males at a time, we decided to track one male at a time to accurately test for speed of the wild type, *tax-2* mutant, and *tax-4* mutant males. We see that *tax-2* and *tax-4* mutant males indeed respond faster than wild-type males to the volatile mating cue (see Figure 10).

To investigate whether *tax-2* or *tax-4* mutants move faster or slower in general, I tracked their movement using WormTracker 2.0 to measure the mean velocity, forward velocity, and backward velocity. *him-5*, *tax-2* and *tax-4* males did not have significantly different velocities (see Figure 11).

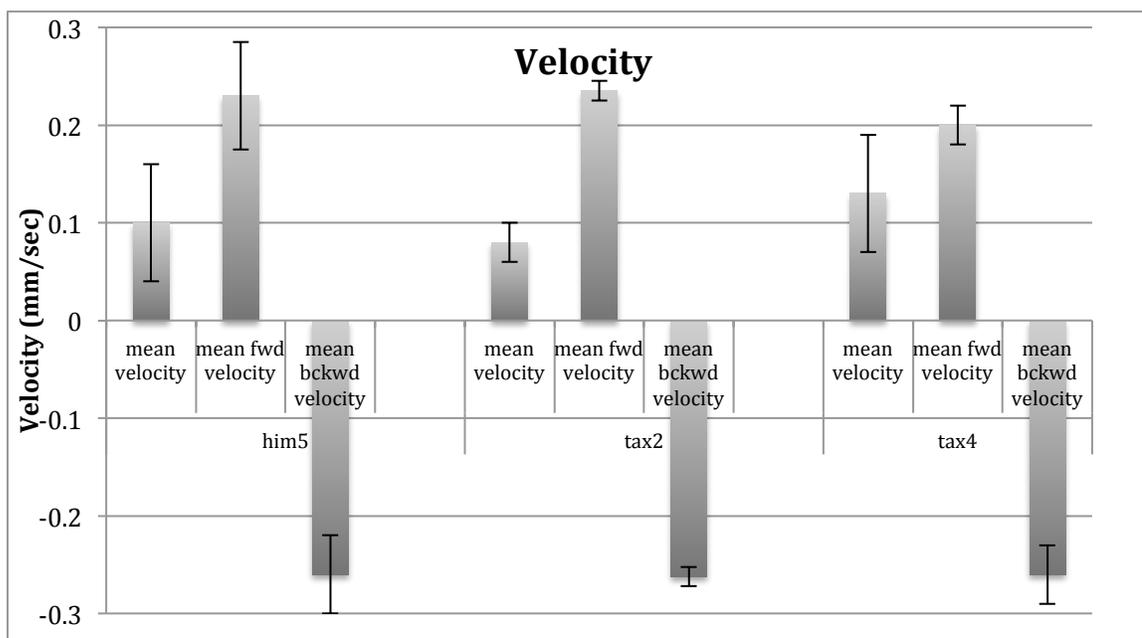


Figure 11: *him-5*, *tax-2*, and *tax-4* males do not have significantly different velocities.

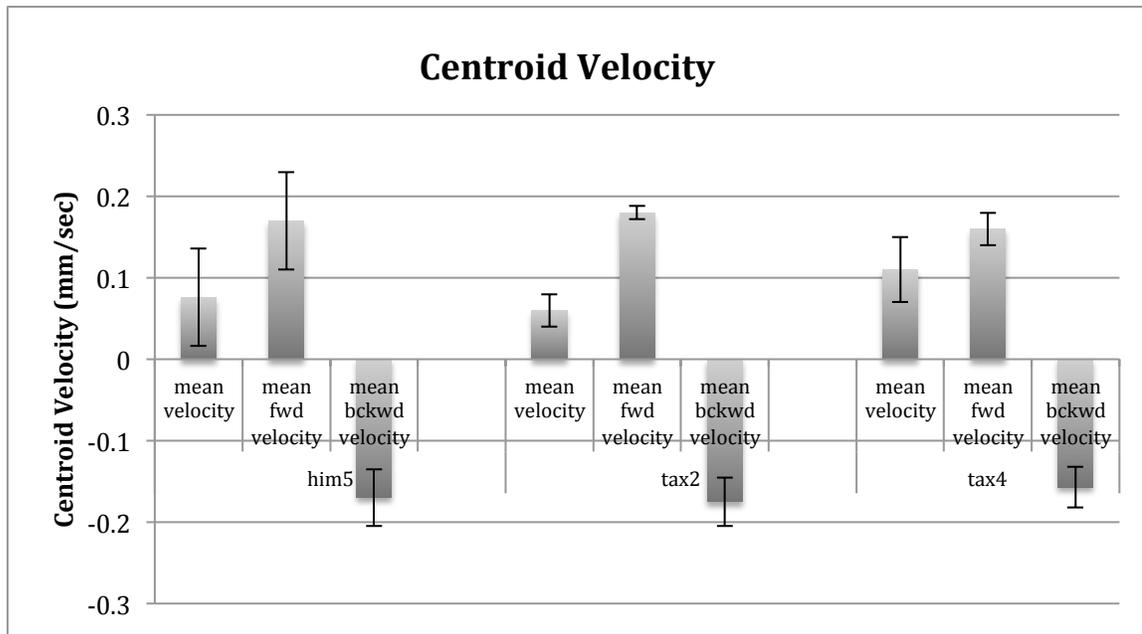


Figure 12: *him-5*, *tax-2*, and *tax-4* males do not have significantly different centroid velocities.

I also looked at centroid velocity because differences in amplitude might reveal differences in actual velocity along a given axis. *him-5*, *tax-2*, and *tax-4* males do not have significantly different centroid velocities (see Figure 12), thus the speed of the different strains did not affect the *tax-2* and *tax-4* males to detect the cue more efficiently. This is interesting because it implies that *tax-2* and *tax-4* play a role in negative inhibition, such that the release of this inhibition allowed for faster location of the volatile cue. Previous studies have shown that many of the neurons that express *tax-2* and *tax-4* are defective in *tax-2* and *tax-4* mutants, including AFD, ASE, AWC, and AWB[11]. Because neither *tax-2* or *tax-4* are expressed in AWA[12], the heightened response to the cue most likely does not act directly via AWA, but perhaps through inhibition from another cell involved in the circuit for sexual attraction. Structural analysis of ASJ in *tax-2* and *tax-4* mutants have revealed that there is a neural outgrowth that could result from axon guidance defect or a

change in the neuronal sensory activity that may indirectly lead to defects in neuron axon outgrowth[11]. These mechanisms might be similar for other cell types, any of which may then interact with AWA or otherwise affect response to the volatile mating cue. These findings warrant a closer look at modulation of sensory neural circuitry, given that there seems to be an interesting interaction between *tax-2*, *tax-4* and inhibition of volatile transduction that has not yet been addressed.

The Aqueous Mating Cue Requires the Sensory Neuron ASK

Chemotaxis to water-soluble attractants require different sensory neurons than chemotaxis to volatile attractants [13] defining distinct classes of gustatory and odorant chemosensory cell types. The 8 pairs of exposed amphid neurons detect water-soluble attractants, with ASE being the major gustatory neuron responsible for chemotaxis to Na⁺, Cl⁻, cAMP, biotin, lysine, and serotonin[14]. ASE-ablated males do not display any defect to the aqueous or volatile cue (see Figures 8 and 9), indicating that ASE does not play a role in detection of the mating cue.

Studies conducted by Srinivasan et al. (2008) were performed using lyophilized hermaphrodite liquid culture[15], which is comparable to our aqueous-only cue. *ascr#3* is one of the compounds found in the fractionation experiment, which elicits sex-specific attraction in *C. elegans* males and fails to elicit attraction in ASK-ablated males[15]. ASK-ablated males also fail to display chemoattraction to our aqueous mating cue (see Figure 11). More specifically, ASK-ablated males enter the aqueous cue-conditioned region and are distinctly repelled. The repelled behavior involves stopping upon contact with the cue-conditioned region, turning at a sharp angle, and moving away from the conditioned area.

Laser-ablated *him-5* males to the aqueous cue

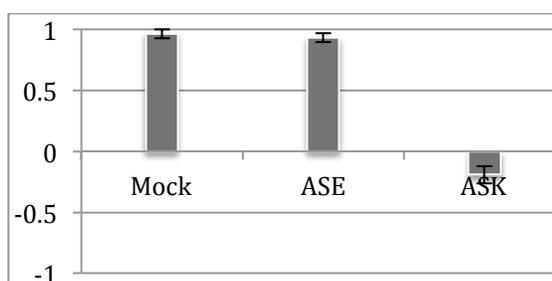


Figure 11: ASE-ablated *C. elegans* males, but not ASK-ablated males, are able to find the aqueous cue

This repellent behavior makes me question the conclusion that ASK carries the receptor for the attractant, given that the possibility exists that ASK may be suppressing a nociceptive behavior, which may explain why ASK-ablated males quickly retreat upon contact with a worm-derived cue.

Because *ocr-1*, *ocr-2* double mutant males are defective in their attraction to the aqueous cue and are only co-expressed in the neurons AWA and ADL, I have also performed ablations on ADL. We do not, however, see any defects in ADL-ablated males towards either the volatile or aqueous cue (see Figures 8 and 9).

Cilia Formation Mutants are Defective in Attraction to the Aqueous Cue

Ciliogenesis is guided via the intraflagellar transport (IFT) of ciliary precursors along the growing ciliary structure[16]. Part of the IFT machinery is encoded by *osm-5*[17] and *osm-6*[18], both of which are necessary for the detection of the aqueous mating cue. We see that *osm-5* mutant males have severely impaired chemoattraction to the aqueous mating cue, but have normal attraction to the volatile cue. The *osm-6* mutant males show defect in

attraction to both the aqueous and volatile mating cue. Simon et al. also found that *osm-5* and *osm-6* mutant males fail to respond to the mating cue[19]. *osm-3* encodes for a motor kinesin[20], without which leaves the cilia without a distal segment[21]. *osm-3* mutant males have no noteworthy defect in attraction to the aqueous or volatile mating cue (see Figures 8 and 9), indicating that the distal segments of the sensory cilia are most likely not necessary for sexual attraction.

Male-Specific Mutants are Not Defective in Chemoattraction to the Cue

Transient receptor potential polycystin (TRPP) channels are required for physical male mating behavior[19, 22] and are encoded by *lov-1* and *pkd-2*[22]. Expression of LOV-1 and PKD-2 are found in the male-specific CEM (cephalic companion) neuron, the hook neuron HOB, and sensory ray neurons[22]. *lov-1* and *pkd-2* males demonstrate full attraction to both the volatile and nonvolatile cue, thus they are not required for chemoattraction to either cue. The Bar homeodomain transcription factor CEH-30 is necessary for the sexually dimorphic survival of the male-specific CEM (cephalic male) sensory neurons[23]. *ceh-30* males demonstrate full attraction to both the volatile and nonvolatile cue, therefore they are not required for chemoattraction to either cue.

Identification of the Volatile Cue using Gas Chromatography Mass Spectrometry

To identify the volatile cue, I have developed a setup to sample odors from a flask with either N2 hermaphrodites or bacteria (see Figure 12). I received valuable advice from Nathan Dalleska at the Caltech Environmental Analysis Center, who recommended the use of Teflon tubing (tygon tubing absorbs volatile odors and may release them in future

sampling events) and thermal desorption tubes for the collection of odors for analysis (Tenax TA®, Sigma Aldrich).

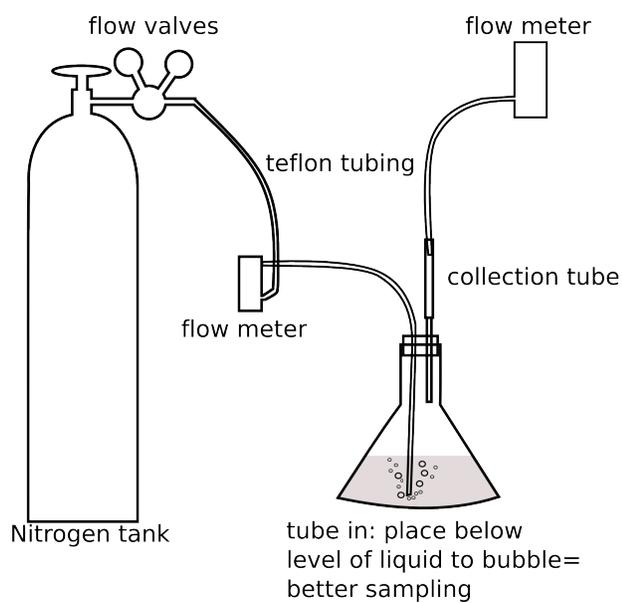


Figure 12: Setup for collection of volatile odors into thermal desorption tube, to be analyzed via GCMS

I begin by testing the following volatile odors:

a) N2 hermaphrodites in S basal (6 incubation incubation, followed by collection)

b) S basal (control buffer) (6hr incubation, followed by collection)

c) 2% w/v *E. coli* HB101 in S basal (6 hr incubation, followed by collection)

The first observation that I made was that when the air bubbled through the N2 hermaphrodites in S basal, the bubbles seemed to travel slower than the bubbles through S basal alone or *E. coli* in S basal. This made me concerned, because I had never considered viscosity as parameter in these assays. It had never occurred to me that the nematodes were producing a viscous secretion, which would inevitably affect the release of volatile odors into the environment, given that viscosity affects the partial pressure of dissolved gases.

GCMS analysis revealed that the N2 hermaphrodites produced a different panel of odors than *E. coli*, however this does not reveal information about what I had been testing in my volatile assay. The appropriate test would be to measure the viscosity of the N2-incubated buffer after 6 hours and then replicate that viscosity in the food control. I standardized the viscosity of the worm-incubated buffer and bacteria-incubated buffer with methyl cellulose, a non-allergenic, nontoxic compound. The results of this experiments revealed that bacterial odors remained in the viscous solution long after the bacteria had been filtered.

I exchanged the original control of my volatile assay control with methyl cellulose-incubated *E. coli* HB101. Males were no longer attracted to the hermaphrodite-derived volatile cue over this food cue.

Here are the conclusions that I draw from these experiments:

1) The Aqueous Cue data are unaffected and remain true, given that the nonvolatile cues were collected without food.

2) The prior belief that N2 hermaphrodites require food to produce a volatile cue is

probably untrue. N2 hermaphrodites simply do not produce a volatile cue. The results reported in this Chapter is most likely a response to a (select) variety of food-derived odors.

3) The control for food + worms is definitely not buffer (as used in White et al. (2007) nor is it simply food (my experiments). *E. coli* HB101 do not produce a viscous substrate and therefore do not trap odors in the same way that N2 hermaphrodites do. Since this time, I have observed differences in viscosity in worm-incubated buffer and have communicated with other lab members that verify that certain species produce more or less viscous substrates (Leighton, personal communication.)

It is with great disappointment that I must report that the volatile studies described in this chapter most likely represent attraction to bacterial food odors. It is still interesting to note that nematodes may secrete this cue in nature for the purpose of sequestering bacteria-derived odors, however such findings must be concluded by a separate study.

Volatile Cues exist in Male-Female species of *Caenorhabditis* but not hermaphroditic species

The follow-up experiment to this discovery is to test for nematode volatile cues without the presence of food. I no longer speculate that food is necessary for the production of the volatile cue and all cues will henceforth be collected in buffer alone. I certainly may be missing a class of food-dependent cues, however I find there to be too many confounding variables. I report that females, but not hermaphrodites are able to elicit volatile attraction from conspecific males and males from other *Caenorhabditis* species (see Figure 13).

Volatile Chemoattraction in the Genus *Caenorhabditis*

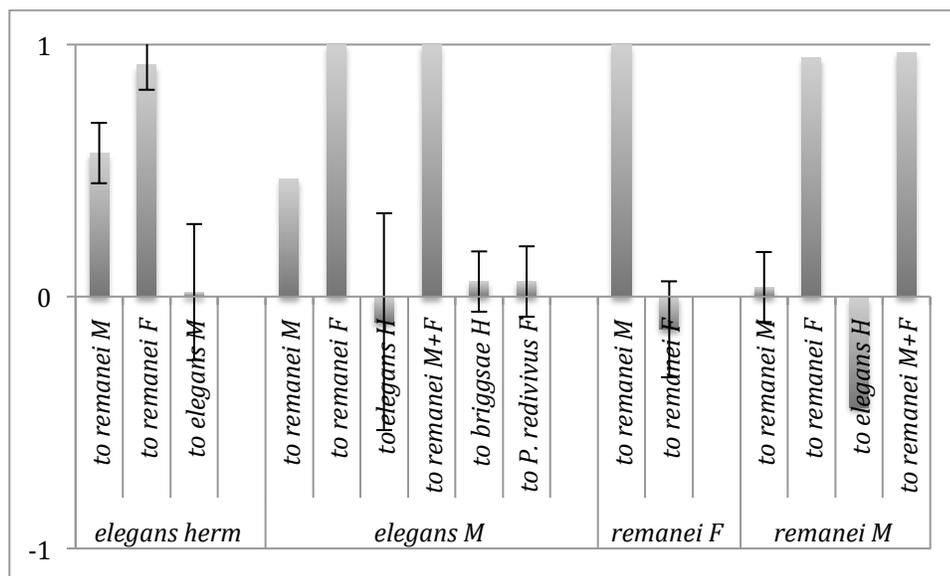


Figure 13: Females, but not hermaphrodites, from the genus *Caenorhabditis* elicit volatile chemoattraction from conspecific males and males from related species.

This is interesting because it confirms my initial suspicion that females, but not hermaphrodites have a greater incentive to produce long-range mate attractants. These preliminary findings warrant a very interesting second look at the source of the mate-finding cue, given that it is now clear that the chemical nature varies between hermaphroditic and female species.

Lastly, I have collected cues from multiple *Caenorhabditis* species and tested these cues on the nonvolatile assay described in Chapter 2. I have found that most species of *Caenorhabditis* are attracted to the nonvolatile cue produced by other species (see Figure

14).

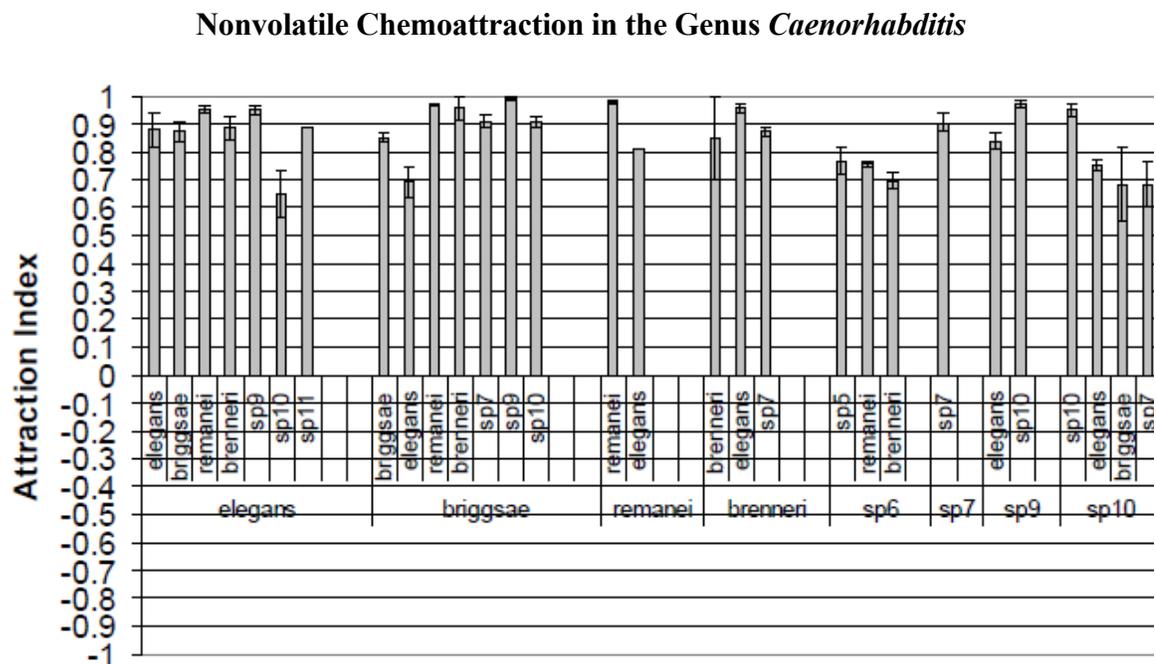


Figure 14: Males from the genus *Caenorhabditis* are attracted to the nonvolatile cues produced by other *Caenorhabditis* species.

These results are interesting because they beg the question of why different species produce a cross-species cue. Perhaps this is a demonstration of altruism in the genus *Caenorhabditis*, such that nematodes might share information about their location and perhaps ideal environmental conditions. It might also be beneficial for species to coexist with other nematodes, given that beneficial collective motion is a trend often seen in nature.

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Chapter 4
Ascarosides are pheromones for both free-living and parasitic nematodes

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Abstract

Nematodes are among the most diverse phyla of animals in the world, occupying virtually every ecological niche. Because nematodes cause many human diseases and create significant agricultural loss, there have been many studies on pheromone-mediated nematode behaviors, but very few pheromones have actually been identified. Here we report, via high-performance liquid chromatography electrospray ionization mass spectrometry, the discovery that many free-living and parasitic nematodes secrete small-molecule pheromones called ascarosides. We show, via video-recorded attraction assays, that multiple genera of nematodes are attracted to distinct but overlapping subsets of ascarosides, revealing cross-species communication. We also report, via activity-guided purification, that the *Panagrellus redivivus* female sex pheromone is also an ascaroside, *ascr#1*, which was first discovered in the model organism *Caenorhabditis elegans* for its small role in stress-resistant diapause. This finding demonstrates that ascarosides play a role in multiple survival strategies that may reveal useful targets for the control of parasitic nematodes.

Introduction

Nematodes are the most abundant animal in the world, by individual count[1]. They have been found to inhabit sulfurous sediment, deep-sea trenches, human lymph nodes, pig intestines, plant roots, whale placenta, arctic ice, and many other ecosystems, making them one of the most successful groups of animals on earth[2-8]. Their ubiquity has led to a number of problems for civilization, including the loss of crops and the spread of neglected tropical diseases[9, 10]. Therefore, many pheromone-mediated nematode behaviors have been studied, which collectively demonstrate that males can successfully chemotax towards females in plant roots, bacterial film, sand, agar, and mammalian intestines[11-23]. There have also been many attempts made at pheromone purification[24], but only two nematode species have had their pheromones successfully purified: *Heterodera glycines* and *Caenorhabditis elegans*. [25-27]. In the soybean-cyst nematode, *H. glycines*, vanillic acid was identified as a component of the female sex pheromone. Since this discovery, chemical analogs of vanillic acid have demonstrated potential to reduce parasitic load in affected crops[28]. Studies in the soil-dwelling model organism *C. elegans* have shown that a family of small-molecule pheromones, called ascarosides, plays a role in both mate finding[26] and entry into dauer, a stress-resistant life stage[29]. These ascaroside studies were among the first to integrate modern advancements in analytical chemistry[26, 30] with the wealth of genetic, cellular, and developmental data that decades of work on *C. elegans* has elucidated.

Because *C. elegans* is just one of over 25,000 known nematodes[9], we aimed to purify pheromones from other nematode species using advanced analytical chemistry that was otherwise not available during the era when most purification attempts were made. We

have taken several approaches to this investigation, starting with the double-blind purification of the female sex pheromone in the sour-paste nematode *Panagrellus redivivus*. We have combined C18 solid-phase extraction, ion exchange, liquid chromatography mass spectrometry, nuclear magnetic resonance, and high performance liquid chromatography with an automated assay that we have developed to score nematode chemoattraction. Here we report the discovery of an ascaroside as the *P. redivivus* female sex pheromone. Because this is the second independent purification of an ascaroside as a nematode pheromone, our finding has led us to hypothesize that ascarosides could be an important nematode cue. To address this possibility, we then collected worm excretions/secretions (E/S) from a wide range of both free-living and parasitic (plant, insect, and mammal) nematodes between Clades 2-12 and performed high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) for rapid detection of ascarosides. Here we report that many nematodes indeed produce a range of ascarosides. Because we have synthesized many of these ascarosides from their initial discovery in *C. elegans*, we were able to test over a dozen ascarosides on multiple genera of nematodes, using the automated chemoattraction assay, to investigate a functional role for these small molecules. Here we show that multiple genera of nematodes are attracted to many of the same ascarosides, revealing that ascarosides are used by nematodes to communicate between species despite the fact that they occupy very different ecologies. This presents a new paradigm in our understanding of nematode communication, as this is the first identification of pheromones produced and used by more than one nematode species.

The *Panagrellus redivivus* female sex pheromone is an ascaroside

We have developed an automated assay to score nematode attraction in an unbiased manner (see Figure 1).

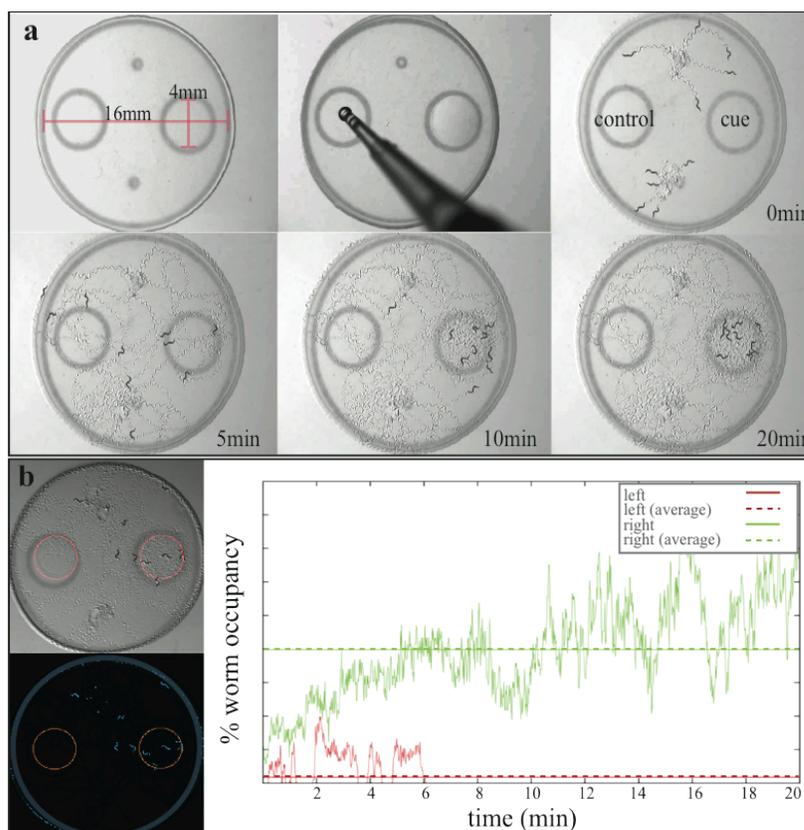


Figure 1: The Attraction Assay **a)** 1-day old 16mm bacterial lawn is grown on a standard 5cm agar plate. A transparent template is attached below the plate to specify regions that will be conditioned and subsequently scored. 0.6µL of the experimental cue is dropped on a 4mm scoring region on the lawn and 0.6µL of the control is dropped in the scoring region on the other side. 10 males are placed at points equidistant from the foci of the scoring regions and the area containing the bacterial lawn is recorded for 20 minutes. **b)** Attraction is measured using an automated program, which computes the worm-pixels to all-pixels ratio in each scoring region for each frame (one frame per second over 20 minutes). The output is a plot of worm occupancy ratio vs. time for each region of interest, providing a metric for comparing time spent in each area.

Using this method, we have been able to efficiently screen for nematode attraction or repulsion to a given cue. This has allowed us to perform a large-scale study of fractionated worm secretions in order to isolate nematode-derived attractants. We chose to isolate the sex pheromone of the free-living nematode *Panagrellus redivivus* because it is easy to

study (grows quickly and moves well) and the males and females have distinct, gender-specific cues (see Figure 2).

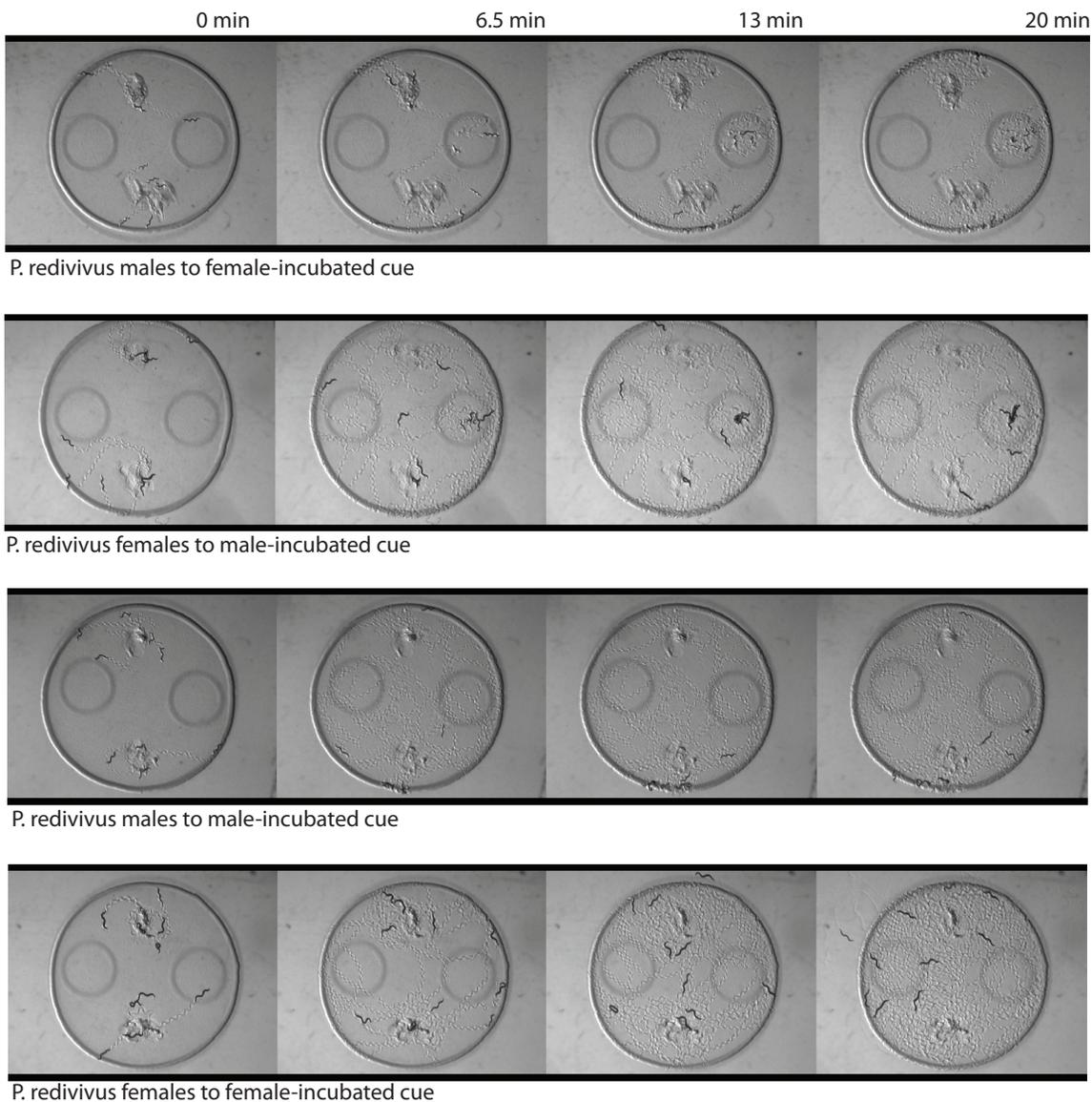


Figure 2: *Panagrellus redivivus* gender specific cue. *P. redivivus* males are attracted to female-incubated, but not male-incubated cue. *P. redivivus* females are attracted to male-incubated, but not female-incubated cue.

We adapted the multistep fractionation scheme (see Figure 3a) described by Srinivasan et al. (2008), starting with C18 solid phase extraction (SPE) of adult *P. redivivus*-incubated water.

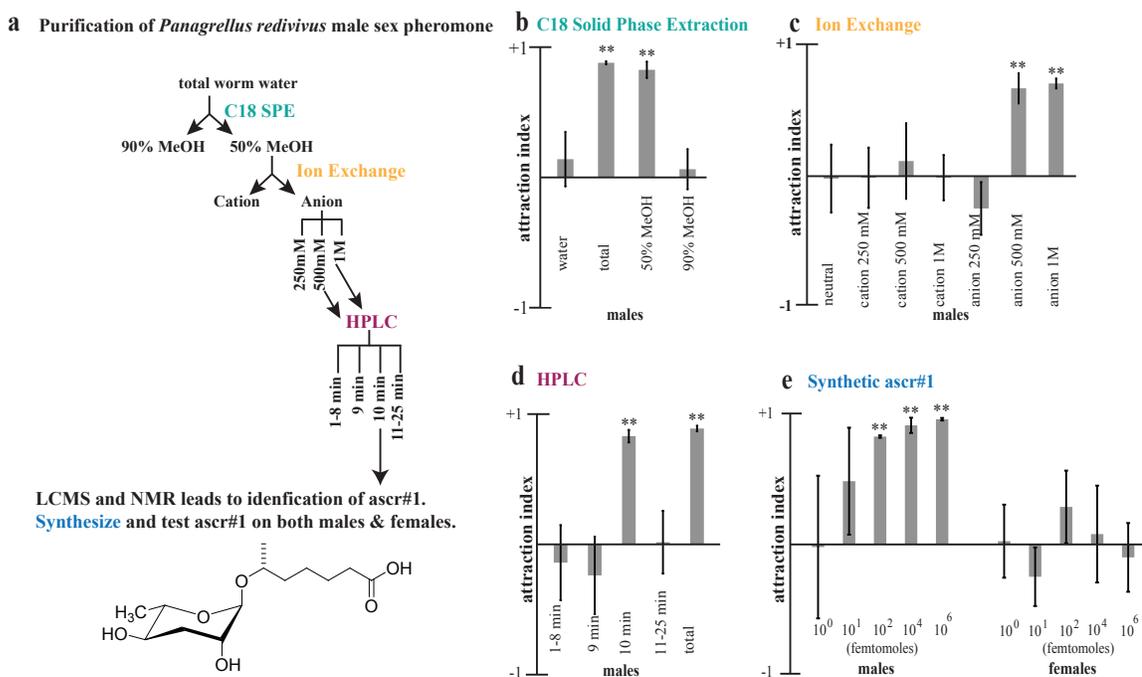


Figure 3: Purification of the male sex pheromone in *Panagrellus redivivus* a, *P. redivivus* were grown in mixed-gender liquid cultures and the total supernatant was fractionated using C18 solid phase extraction, ion exchange, and high performance liquid chromatography. Male attraction was measured via the Attraction Bioassay (see Figure 1). b, The total worm water was divided using 50% MeOH and 90% MeOH extractions via C18 SPE, with significant male attraction to both the combined fractions (total) and 50% MeOH fraction. c, The 50% MeOH fraction was subdivided using ion exchange, with no male attraction to the cation fractions and significant attraction to both the 500mM and 1M anion fractions. d, The 500mM and 1M anion fractions were further subdivided using HPLC. The compound eluted at 10 minutes produced full male attraction, which was then identified as ascr#1 using liquid chromatography mass spectrometry (LCMS) and nuclear magnetic resonance (NMR). e, ascr#1 was then synthesized and tested at different doses, demonstrating that males were attracted to ascr#1 at concentrations between 10² fmol and 10⁶ fmol whereas females were not attracted to ascr#1 between 10⁰ and 10⁶ fmol. Error bars, S.D. P values were determined using Student's t-test. ** P < 0.01.

P. redivivus males tested on the Attraction Assay were significantly attracted the region conditioned with the 50% MeOH fraction (see Figure 3b), which was then fractionated further using ion exchange. The ion exchange yielded three anion fractions (250mM, 500mM, 1M) and three cation fractions (250mM, 500mM, 1M) of which the 500mM anion and 1M anion fractions elicited significant male attraction (see Figure 3c). These were further fractionated using high-performance liquid chromatography (HPLC), which

produced a component at 9 min and another component at 10 min. Males were significantly attracted to the component at 10 min (see Figure 3d), identified as (-) 6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yl)oxy heptanoic acid (aka ascr#1, C6[31] or daumone[27]) using both nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LCMS) (see Figure 3a).

We tested synthetic samples of ascr#1 to confirm activity and saw that *P. redivivus* males were attracted to synthetic ascr#1 at 10^2 fmol, 10^4 fmol, and 10^6 fmol (see Figure 3e). The monophasic response to increased doses of ascr#1 is different from the biphasic response observed in *C. elegans* male attraction to ascr#2 and ascr#3, and the biphasic response of *C. elegans* dauer formation to increased doses of an indole-containing ascaroside[32]. However, this monophasic trend has also been observed in dauer formation to increased doses of ascr#2, ascr#3, and ascr#5[29], alluding to the complexity of ascaroside-mediated behaviors.

We tested female *P. redivivus* to verify gender-specificity and found that they were not attracted to ascr#1 between 10^0 and 10^6 fmol (see Figure 3e), suggesting that ascr#1 is indeed the male-specific attractant. Although females are not significantly repelled by ascr#1 by measure of the Attraction Index, further inspection of the videos revealed that females tended to stall upon entering the region holding ascr#1, followed by a change in direction and abrupt exit of the region. This exit from the ascr#1-conditioned region involved a delay that made their time spent in the experimental region comparable to the control region, thus hindering a repulsive score on the Attraction Index. We thus used a separate method for scoring repulsion that focused on behavioral response rather than time spent in each region. We define repulsion as the immediate exit of the conditioned region

before entering a full worm's length. The results indeed revealed that females were significantly repelled by ascr#1 at 10^6 fmol when compared to the control region (see Supplemental Figures 1 and 2). Therefore, ascr#1 is a *P. redivivus* female sex pheromone, which attracts males and repels females at a concentration of 10^6 fmol.

This result is surprising because we chose a nematode species from a different Clade than *C. elegans*[33], towards the goal of characterizing different sex pheromones in a comparative model organism. However, independent purification of pheromones from both *C. elegans* and *P. redivivus* has led to the discovery of ascaroside sex pheromones. Also, ascr#1 has already been characterized in *C. elegans* for its small role in dauer induction,[27] demonstrating that the same pheromone could be used for different purposes between species. Ascarosides can also play different roles within species: ascr#1 both attracts *P. redivivus* males and repels *P. redivivus* females, while they mediate both mate finding and dauer formation in *C. elegans*.

Ascarosides are broadly present in many nematodes

To further investigate the general role of ascarosides, we screened for ascarosides in a wide range of nematodes. We included nematodes that had a broad range of lifestyles, including both parasites (plant, insect and mammal) and free-living nematodes. We also sampled between ancestral nematodes (Clade 2) and contemporary nematodes (Clade 12)[33].

Specifically, nematodes were incubated in medium, filtered out, and the conditioned medium analyzed by HPLC-ESI-MS analysis (see Figure 4a). Nineteen different ascarosides, of which 13 represent new structures previously identified in media extracts of

C. elegans, were identified by analysis of MS ion traces and comparison of the HPLC retention times (see Figure 4b).

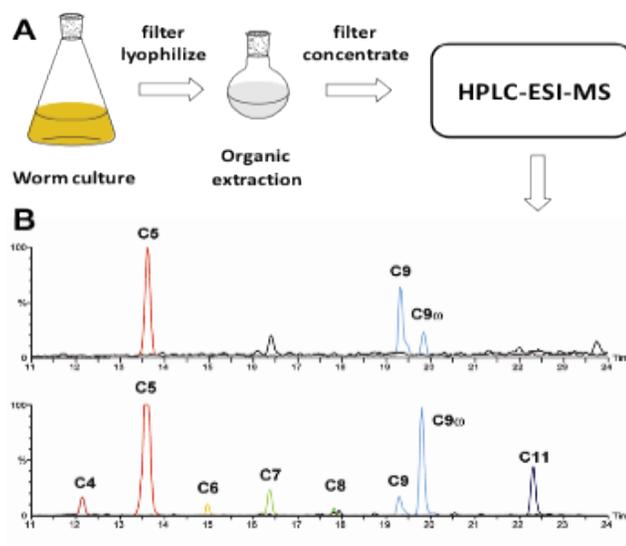


Figure 4: HPLC-ESI-MS analysis of worm extract. a, Preparation of worm media extracts for HPLC-ESI-MS analysis. b, Stage specific biosynthesis of ascarosides in *S. carpocapsae*; Infectious juveniles release small amounts of ascr#9, ascr#10, and omas#10 while adults release a complex mixture of ascarosides.

We have found that most nematodes produce ascarosides (see Figure 5). We observed a range of unique and overlapping ascaroside production. For example, a diverse range of nematodes produced ascr#10, including the rat parasitic nematode, *Nippostrongylus brasiliensis*; the entomopathogenic insect nematodes, *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *Steinernema glaseri*, and *Steinernema riobrave*; the soil nematode, *C. elegans*; and the necromenic insect parasites, *Pristionchus pacificus* and *Koernia sp.* Some of the nematodes (*Oscheius tipulae*, *Pelodera strongyloides*) produced few or none of the known ascarosides.

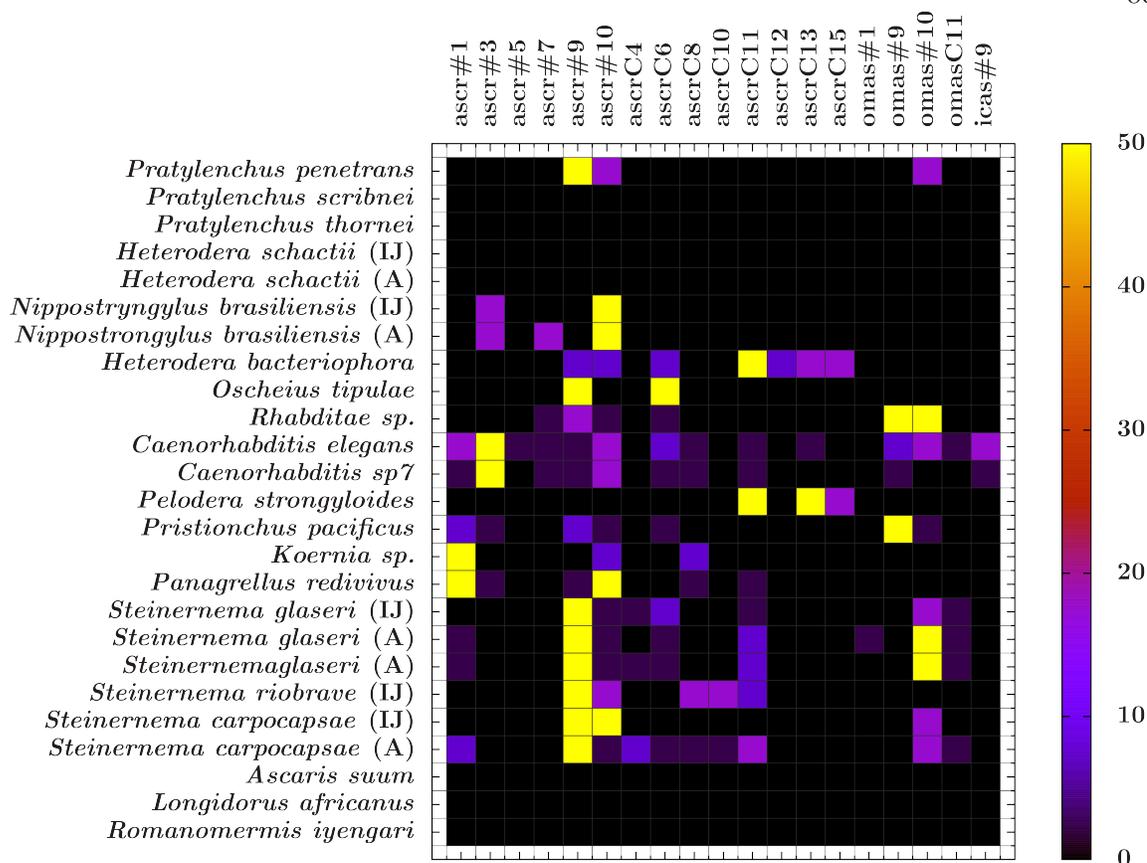


Figure 5: Ascarosides are broadly present in a wide range of nematodes. Worms were grown up in large cultures, washed several times, and incubated for 6 hours at 1 worm/ μ L. Parasitic species indicated as infective juveniles (IJ) and adults (A) were collected separately, otherwise cultures contained mixed stages. Worms were then filtered out and the remaining supernatant was screened for the presence of ascarosides previously found in *C. elegans*, using LCMS. *P. penetrans*, *P. scribnei*, *P. thornei*, *H. schactii*, *N. brasiliensis* (IJ), *H. bacteriophora*, *S. glaseri* (IJ), *S. riobrave* (IJ), *S. carpocapsae* (IJ) and *R. iyengari* were incubated in ddH₂O. *N. brasiliensis* (A) was incubated in .15M NaCl. *O. tipulae*, *Rhabditae* sp., *C. elegans*, *P. strongyloides*, *P. pacificus*, *Koernia* sp., *P. redivivus*, *S. glaseri* (A), and *S. carpocapsae* (A) were incubated in *S. basal*. *A. suum* was incubated in DMEM. Colors represent different % relative quantity of ascarosides within species.

The highly potent *C. elegans* hermaphrodite attractant icas#9 [Srinivasan et al., submitted], which has an indole-3-carbonyl-unit attached to the 4-position of the ascarylose ring, was also detected in *Caenorhabditis* sp7. *Heterorhabditis bacteriophora* and *P. strongyloides* were dominated by long chain ascarosides showing 11-, 13-, and 15-numbered carbon side-chains. Ascarosides with saturated 5-, 7-, 9-, and 11-numbered side chains from *C. elegans*, *Rhabditae* sp., *Pristionchus pacificus* and *Steinernema* sp. media extracts were

accompanied by additional later eluting isomeric compounds showing signals at m/z 247, 275, 303, or 331, suggesting the presence of omega-oxygenated derivatives. The structure of these *omega*-oxygenated ascarosides called “omas” was proven by synthesis of two representative components featuring pentanoic acid and nonanoic acid side chains, omas#9 and omas#10 (see Figure 6a).

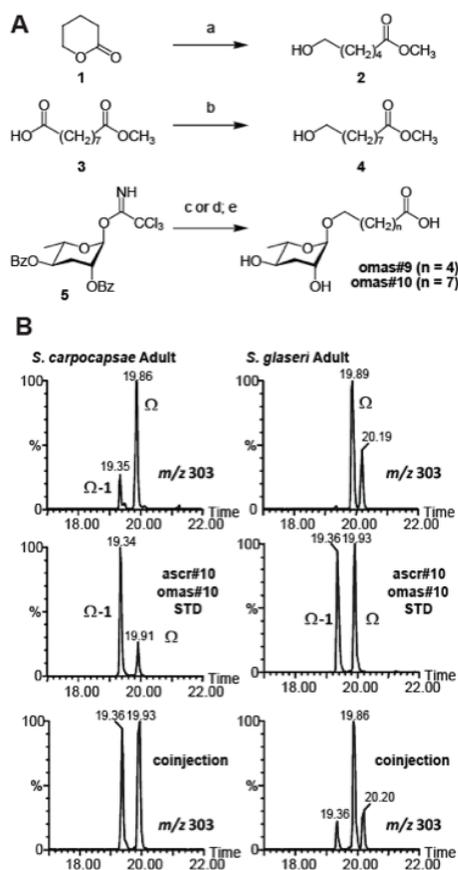


Figure 6: Identification of omega-oxygenated ascarosides. a, Synthesis of omas#9 and omas#10; a: CH₃OH, cat. H₂SO₄, 100%; b: BH₃, THF, XX%; c: TMSOTf and 2 in DCM, XX%; d: TMSOTf and 4 in DCM, XX%; E: LiOH, THF, XX%; b, Identification of omas#10 in *S. carpocapsae* and *S. glaseri* using coinjection with a synthetic standard of ascr#10 and omas#10. The signal showing m/z 303 at 20.20 min in *S. glaseri* media extracts remained unidentified.

Acid catalyzed methanolysis of δ -valerolactone **1** yielded methyl 5-hydroxypentanoate **2** [Huchstep & Taylor 1982]. BH₃ reduction of monomethyl azelate **3** yielded methyl 9-

hydroxynonanoate 4. These omega-hydroxyl methylesters were linked to 2,4-di-*O*-Bz-protected ascarylose. Alkaline hydrolysis and column chromatography afforded pure samples of omas#9 and omas#10 identical to the natural products (see Figure 6b). The new omega-oxygenated ascarosides, omas#9 and #10 represent the long chain derivatives of the potent dauer-inducing ascr#5 and indicate that two series of homologous side chains are found in ascarosides from nematodes.

Developmental stage specific ascaroside production was evident for *S. carpocapsae*; infectious juveniles produced only ascr#9, ascr#10 and omas#10, whereas adults released a complex ascaroside mixture showing fatty acid derived side chains from C₅ – C₁₃ (see Figure 5b). Along with the odd numbered sidechains (C₅, C₇, C₉, C₁₁, and C₁₃), smaller amounts of the corresponding even numbered homologs (C₆, C₈, C₁₀) were also detected, indicating that odd and even numbered fatty acid precursors enter the peroxisomal β -oxidation pathway to ascaroside components. Furthermore, the ratio of (omega-1)-oxygenated ascr#10 to the corresponding omega-oxygenated derivative omas#10 was reversed upon development to the adult larval stage. These indications of stage-specific production of ascarosides support the idea that ascaroside mixtures have very diverse functions.

We have also tested for the presence of ascarosides in *Panagrellus redivivus*, given that the previously described purification was limited to activity-guided fractionation and we wanted to verify that the female sex-pheromone, ascr#1, would be identified using this method. Ascarosides ascr#1 and ascr#10 were readily identified as dominant components in *P. redivivus* females, but were not found in males. This finding supports our conclusion

that ascr#1 is indeed a female sex pheromone. We have addressed the discovery of ascr#10 by testing both males and females to several concentrations of ascr#10. Females do not respond to ascr#10 at a concentration of 1nM, 1 μ M, and 1mM; whereas males respond to ascr#10 at a concentration of 1mM but not 1 μ M or 1nM. Because the highest concentration tested during the purification was equivalent to 1 μ M, we believe this is why ascr#10 was not discovered during the activity-guided fractionation. These results suggest that ascr#10 is also a female sex pheromone, but is required in a higher concentration to elicit male attraction.

Different species of nematodes respond to ascarosides

To further investigate the biological function of ascarosides, we have scored attraction of different nematodes to different ascarosides at a range of concentrations (1nM, 1 μ M, and 1mM) on the same bioassay used for the activity-guided fractionation of the *P. redivivus* sex pheromone. Nematodes that were chosen for this study had to fulfill several requirements: moves well and does not have a tendency to aggregate, easy to grow and maintain, and distribution across the assay lawn is even and reproducible. We chose to study males from different species, because males tended to move much more evenly across our bioassay lawn than females (with the exception of *P. redivivus* females, which were therefore included in this study.)

We have found that many of the nematodes are attracted to the same ascarosides, particularly ascr#1, ascr#3, ascr#7, ascr#8, ascr#9, and ascr#10 (see Figure 7 and Supplemental Figure 3). Several nematodes respond to different concentrations of the same ascaroside, for example *P. redivivus* males are attracted to 1mM and 1 μ M of ascr#7 whereas *C. elegans* males only respond to 1mM. This suggests that there are different

Discussion

Our findings suggest that ascarosides could be a universal nematode cue, given their widespread production and recognition by free-living and parasitic nematodes. These findings are evocative of inter-species quorum sensing in bacterial pheromones, which are both produced and sensed by many Gram-negative and Gram-positive bacteria[34]. For the same reason that there has been controversy regarding the uncertainty of shared motives for quorum-sensing bacteria[35], we cannot endorse a unanimous motive for ascaroside production or recognition. In fact, it is clear that ascarosides are used for different reasons both between and within species, given that *ascr#1* plays a small role in *C. elegans* dauer formation and since *ascr#1* attracts *P. redivivus* males and repels *P. redivivus* females. We believe that *ascr#1* is the major female sex pheromone for *P. redivivus*, but recognize the possibility that it could be used by another species for any number of reasons, such as predation, food sharing, coordinated defense, cohabitation, diapause, mate-finding, aggregation, etc.

It is known that Gram-negative bacteria produce N-Acyl homoserine lactone (AHL) pheromones for both intra- and inter-species communication. AHLs are composed of the same homoserine lactone but have species-specific variations in the N-acyl chain[36]. Ascarosides are organized in a very similar fashion; they are composed of the same ascarylose sugar ring but have variations in the attached fatty acid tail[37]. We believe this similar-but-different organization of pheromones helps species to communicate with other species in order to mount a coordinated response when the outcome is mutually beneficial. This multilingual system might not always be beneficial when nematodes might need to communicate within their own species without revealing their motives to others. For this

reason, a species-specific cue seems advantageous. However, unlike AHLs, the same ascarosides are produced by many species of nematodes. Perhaps intraspecific discretion is achieved by the production of a unique blend of ascarosides and/or the existence of different response thresholds. Our findings support this possibility, given that nematodes produce different relative quantities of ascarosides and sometimes respond to different concentrations of the same ascaroside. Because recombination is a theme often used in nature to achieve variation, it is not unlikely that nematodes secrete combinations from the same repertoire of ascarosides to present a specific chemical signature to their surrounding environment.

METHODS

Attraction Assay OP50 *E. coli* is grown on a standard 5cm agar plate (made with standard Nematode Growth Medium). The bacterial lawn is 16mm in diameter and is grown overnight at 20°C before being used in trials. Two 4-mm spots (0.6µL) were placed on opposite sides of the bacterial lawn (using a transparent template to guide spot placement) and several minutes elapsed for the liquid to settle in before placing nematodes down on the assay. Recording began immediately upon worm placement. 0.6µL of the control was placed on one side of the lawn and 0.6µL of the experimental cue was placed on the other side of the lawn, changing the location of the cue throughout trials, between left/right and top/bottom to avoid bias. Nematodes were isolated by gender at the L4 stage the day before being used in trials as developed adults. Five worms were each placed at two points equidistant from the foci of the scoring region (ten total). Trials were recorded for 20

minutes and frames were collected for analysis at 1 frame per second. Results were averaged from at least three different trials. For every nematode species in this study, we tested different total number of worms (using water in both scoring regions) to determine the minimum number of worms necessary for consistent unbiased results over a 20-minute trial. The total number of worms used in the multiple species assays depending on that species' optimal parameters. 10 worms were for *P. redivivus* males and females, 20 worms were used for *C. elegans* males, *O. dolichuridae* males, and *C. sp7* males and 14 worms were used for *S. glaseri* males.

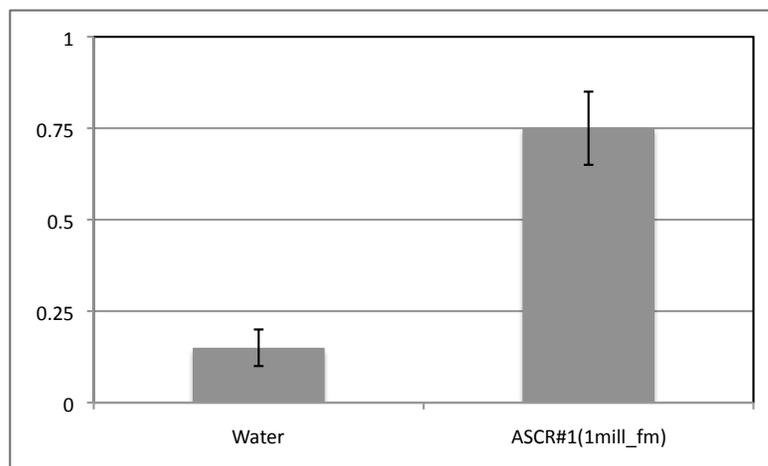
Automated Software A video camera attached to the microscope produces a digital video stream, which is then analyzed. The ratio of time the average worm spends in each region of interest is calculated for every trial. For ease of implementation, we assumed that all worms in a single experiment are roughly the same size. We thus counted worm pixels instead of whole worms, allowing us to take into account fractions of a worm in the region of interest. It also eliminated the need for a shape-based worm identification algorithm, and allowed each frame to be analyzed independently. We applied a band-pass filter to each frame to eliminate the effect of uneven lighting and also accentuate the worms against the background. The worm was then identified after thresholding the filtered image. Throughout each experiment, we know the locations and sizes of the regions of interest. Through the filtering described above, we know which pixels are occupied by worms and which ones are not. We were then able to calculate the ratio of worm-pixels to all pixels inside the region of interest to produce the worm-occupancy ratio. This is done for every frame, giving us a plot output of worm-occupancy ratio vs. time for each region.

Ascaroside Screen. Adult and mixed-population nematodes were incubated at 1 worm/ μ L (with a total volume of 15-50mL) for 6hrs in a 20°C shaking incubator. Infectious juveniles were incubated at 1 worm/ μ L (with a total volume of 15-50mL) for 2 days in a 20°C shaking incubator. Nematodes were filtered out using a 20 μ m polystyrene filter and the worm water was then lyophilized, extracted with methanol, and concentrated in vacuo. The residue was taken up in 150 μ L methanol, filtered, and submitted to HPLC-ESI-MS analysis. Indole-3-carbaldehyde (m/z 144 [M-H]), which was most regularly detected in worm media extracts, was employed as an internal standard.

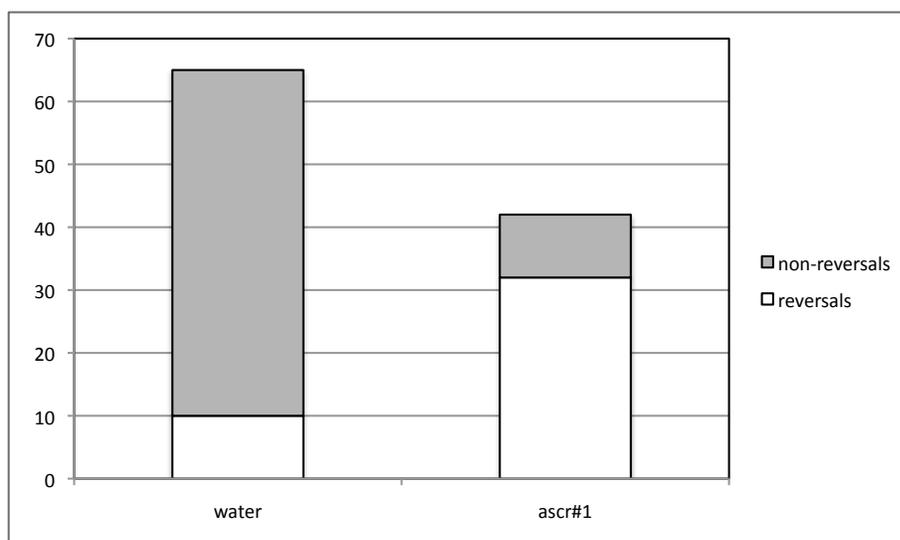
Dauer Assay We adapted the dauer assay from Butcher et al. (2008) and used liquid cultures instead of agar plates. Nematode embryos were synchronized by bleaching twice (3 hours apart) and collected in S complete medium (Brenner, 1974) for liquid culture. They were grown in 4mg/mL HB101 *E. coli* at a density of 6 worms per μ L, along with no ascarosides or 220nM of the ascaroside. They were incubated at 20°C for 4 days, after which they were scored for % dauer formation using observation of anatomical changes and 2% SDS survival tests. Several hundred worms were scored for each trial, with an average of at least three trials.

Acknowledgments We thank Ed Platzer, Robin Gasser, Teresa Mullens, Jennifer Becker, Scott Edwards, John Darsaw, Adler Dillman, and Hillel Schwartz for their helpful discussions and contribution of nematodes to this study. This work was supported in part by a National Institutes of Health grant (GM088290 to FCS and GM085285 to ASE, FCS, and PWS), and the Howard Hughes Medical Institute, with which PWS is an Investigator.

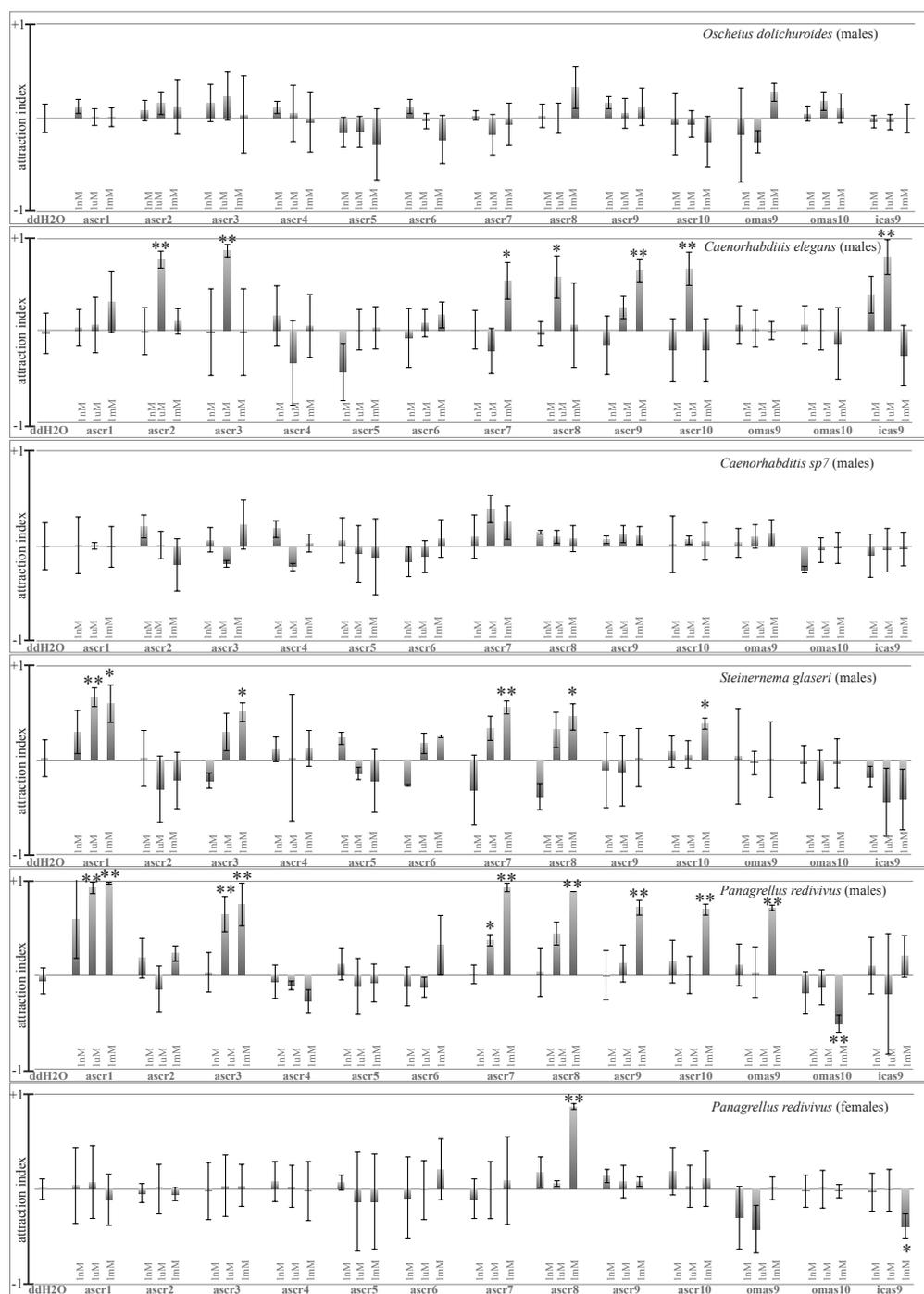
Supplemental Figures

***P. redivivus* females are repelled by ascr#1**

Supplemental Figure 1: Average reversal per trial (n=3) of females upon entering the scoring region.

***P. redivivus* females are repelled by ascr#1**

Supplemental Figure 2: Total reversals from 3 trials of females upon entering the scoring region.



Supplementary Figure 3: Nematodes respond to ascarosides through attraction and repulsion.

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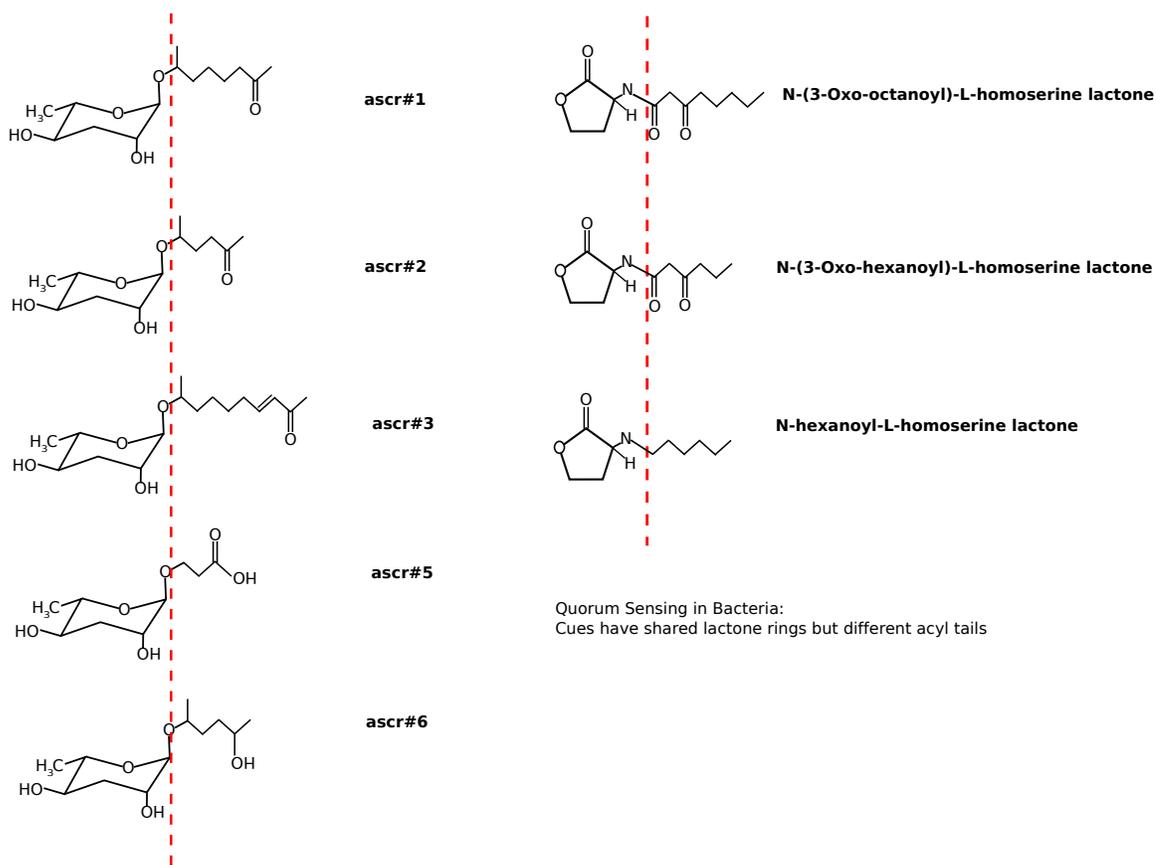
Chapter 5
Conclusions

Given that nematodes are the most abundant phylum of animal in the world (by individual count) and are responsible for causing devastating human disease and agricultural damage (~\$100 billion/year), it is surprising that few nematode pheromones have been identified. I attribute this to the steep convergence of interest on *C. elegans* over the past few decades. Accordingly, *C. elegans* is one of two species from which pheromones have been identified. The other species is the soybean cyst nematode, *Heterodera glycines*, which is the most economically important soybean pathogen for most parts of the world (including the United States) [1].

Because pheromones mediate important social and developmental events, they may be utilized to regulate these events and alleviate crop destruction (as is already being implemented with the *H. glycines* sex pheromone[2]) or disability in third world countries suffering from nematode-borne diseases. I would be cautious, however, of long-term effects that may be devastating to ecologically important nematodes or other invertebrates that might respond to the same pheromones. I am reminded of the Colony Collapse Disorder of honeybee populations, which some speculate is attributed to the accidental windborne spread of neonicotinoids [3]. This gives me some anxiety, as I believe that the utility of behavioral and developmental modifiers may be profoundly beneficial but also hazardous given unforeseeable (and some foreseeable) repercussions. The restricted use of nematode-specific developmental modifiers seems the safest method of parasitic load reduction, perhaps in confined spaces such as pit latrines.

The discovery of general nematode pheromones also presents an opportunity to study evolutionary relationships between nematodes. Many nematodes produce and respond to species-specific (but partially overlapping) blends of ascarosides. This semiochemical

system is similar to the quorum sensing system shared between many species of bacteria, which secrete and respond to different blends of N-Acyl Homoserine Lactones (AHLs). Both sets of semiochemicals utilize this similar-but-different organization of pheromones; all ascarosides have an ascarylose sugar moiety with a variable lipid tail and all AHLs have a lactone ring with a variable acyl tail (see Figure 1).



Quorum Sensing in Nematodes:
Cues have shared sugar rings but different lipid tails

Figure 1: Quorum sensing molecules in nematodes and bacteria utilize a similar-but-different structure. All ascarosides have an ascarylose sugar moiety and all AHLs have a lactone ring.

Perhaps this design is useful to modify signals with limited cost, allowing nematodes to produce a variety of cues when needed. Amendment of preexisting ascarosides may help to

produce communicative signals quickly during times when swift response is necessary.

We must also consider the life of a worm and what benefits they might gain from producing pheromones (intra-specific cues) or allelochemicals (inter-specific interactions), perhaps to share food sources with genetic relatives, hinder competitors from finding limited resources, or cooperate with other species to mount coordinated responses to predators, among many other motives that may be mutualistic, altruistic, or parasitic in nature. We know that ascarosides can function as sex pheromones, epidietic pheromones (dauer formation as a result of high population density) within their own species, however it is unclear how these ascarosides affect recipients from other species without further investigation of interspecies interactions.

Despite the fact that nematodes seem to navigate through life on autopilot, they make subtle decisions to move left or right, up or down. This might seem insignificant on agar plates but is in fact important for chemotaxis towards compromised root structures or epidermal pores, allowing for successful entry into a host and subsequent lifetime of infection. Small amounts of pheromones can influence such decisions, which may be even more effective when amplified by other nematodes that are equipped to receive and relay these messages. These are the trademark benefits of quorum sensing, which utilize positive feedback loops and synchronized group activity. Quorum sensing may not always be advantageous, as strict adherence to group consensus may lead to a fatal decision.^{§§} Nematode species produce a unique set of ascarosides that sometimes overlap but differ in

^{§§} I call this phenomenon “12 Angry Men-tality”.

relative quantity, perhaps allowing tentative language barriers that might be beneficial during times when cross talk is mutually disadvantageous.

I would argue that most animal societies follow similar rules, in which cultures have unique languages, yet adhere to a common set of universally recognized signals. In the case of nematodes, I would not be surprised if animals from other phyla can eavesdrop on ascaroside cues and use this knowledge for their own gain (and perhaps retributive loss). In fact, cross-phyla eavesdropping is likely, given that over 16,000 parasitic nematode species have been described in a broad range of plants, insects, and animals[7].

Humans are not exempt from this affiliation as nematodes continue to plague the lives of millions of individuals suffering from devastating diseases such as lymphatic filariasis, onchocerciasis, ascariasis, trichuriasis, and hookworm disease[8]. Many of these diseases have disappeared from industrialized parts of the world as societies have developed better living conditions and improved sanitation[8]. However, helminthic parasites are thought to impart several advantages based on the hypothesis that chronic exposure to intestinal infections may help to suppress inflammation. Studies have shown that the incidence of inflammatory bowel disease (IBD), such as Crohn's disease, is highest in well-developed countries[9] while epidemiologic studies in sub-Saharan Africa report a very low incidence and prevalence of IBD[10]. Further investigation of this matter has led to the discovery that helminthic nematodes make excretory/secretory (E/S) products that modulate mammalian immune systems, partially through the stimulation of immunoregulatory cell populations, such as regulatory T cells and alternatively activated macrophages[11]. Based on mounting evidence from similar studies and the brave self-clinical trial of an individual who infected himself with the human whipworm, *Trichuris trichiura*, to treat his symptoms of ulcerative

colitis[12], the U.S. Food and Drug Administration has recently granted the pig whipworm, *Trichuris suis*, the status of Investigational New Drug for the use of helminthic therapy[13].

As seen in countless examples in nature, it is the careful balance, rather than the all-or-none relationship between cells, tissues, organs, and organisms that dictates the relative health and wellbeing of an individual. I understand the desperation to find cures for human diseases and I can appreciate the myopic vision of institutions and pharmaceutical companies with singular goals, however I suspect that we are too-often neglecting the bigger picture. I believe that the course laid out before us as physicians and scientists will gain greater illumination through the lens of evolution and ecology, from which I hope we may all continue to gain a greater perspective.

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