April 2019

Sex Pheromone Attractive Response in C. elegans and Related Species

Jane Axi Lockery
Worcester Polytechnic Institute

Sex Pheromone Attractive Response in *C. elegans* and Related Species

A Major Qualifying Project Report
Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the Degree of Bachelor of Science
by:

Jane Lockery

Date: April 25, 2019

Approved by:

Jagan Srinivasan, PhD
Biology & Biotechnology

Table of Contents

Abstract

The nematode *Caenorhabditis elegans* uses pheromones as a primary means of communication. One pheromone is ascaroside #8 (ascr#8), which is secreted by hermaphrodites to attract males. Since pheromones can be effective in closely related species, I performed behavioral assays to investigate whether an attractive response to ascr#8 would be seen in the other two hermaphroditic species in the *Caenorhabditis* genus*, C. tropicalis* and *C. briggsae*. My data shows differences in the levels of attraction to ascr#8 among the three species, implying that the response is unique to *C. elegans*.

Background

Pheromones are a means of chemical communication between organisms

In living organisms, communication between individuals is extremely important. Various signals can help entire groups to locate food, escape from danger, protect their territory, and attract mates, among other actions (Michigan State University, 2008). Communication can be visual, auditory, tactile, or chemical (or less commonly by other means such as electrical or sonal) (Michigan State University, 2008). Chemical communication, also known as olfactory communication, involves biological substances called pheromones.

A pheromone is an endogenous compound produced and secreted by an individual which induces a specific reaction in another organism of the same species (Karlson, Luscher 1959). In some cases, members of closely related species may also be affected. The reaction induced may be behavioral or developmental in nature (Karlson and Luscher, 1959). Pheromones can also be sex-specific, meaning that they are secreted by only one sex and elicit a reaction only in members of the opposite sex (Shorey, 1973). Most pheromones are detected by scent (or chemosensation), in which sensory neurons detect the odorant in the environment and the attached nerve sends a signal to the brain (Wyatt, 2015).

Many organisms use pheromones. The first pheromone discovered was in silkworm moths. Female moths produce bombykol, which is sensed by male moths via receptors in their antennae, resulting in an attractive response (Sandler et al., 2000). Some aquatic creatures use pheromones as well. The Western Pacific anemone, *Anthopleura elegantissima,* releases an alarm signal called anthopleurine when injured, and when conspecifics sense the molecule, they contract in attempt to protect themselves (Puyana, n.d.).

Several mammals also use pheromones. For instance, cats release pheromones from several places on their bodies, including the face. Synthetic versions of feline facial pheromones are available for behavioral therapy in cats that struggle with aggression or anxiety (DePorter, 2016). There has even been research into the presence of human pheromones. In one study, women smelled shirts worn by men, and the researchers found that women tended to be more attracted to shirts of men with an immune system makeup that was different from their own (Particularly, they were attracted to shirts of those who had a differing MHC-1 complex.) (Wedekind et al., 1995) (Wyatt, 2015). However, this and similar studies generally have small sample sizes and probable bias, and they have not had results replicated (Wyatt, 2015).

In order to identify a pheromone, a molecule must be identified, isolated, and synthesized and then tested to see if it alone produces a particular behavioral or psychological response (Wyat,, 2015). Pheromone research in humans is also confounded humans' consciousness and their ability to make choices, which would make it even more challenging to study responses that occur without any bias. Therefore, studying the brain is difficult due to its inherent complexity. To work around this issue, researchers use *C. elegans* as a model organism.

Caenorhabditis elegans as a good model organism

Caenorhabditis elegans is a transparent, nonparasitic nematode about 1 mm in length (Figure 1). A member of the phylum *Nematoda*, it has a smooth, unsegmented body that tapers at the ends. It is found worldwide in soil and rotting vegetation, where it feeds on bacteria (University of Minnesota, n.d.) (Felix et al., 2014). *C. elegans* does not possess the senses of hearing or sight; instead relying on its senses of touch, taste, and smell to take in information about its surroundings (Corsi et al., 2015). Its objectives in its two to three-week lifespan are simple: feed, mate, and reproduce.

Figure 1: *Caenorhabditis elegans* adult. Average length is 1 mm. (Universiteit Utrecht)

As primitive as *C. elegans* may seem, it shares many fundamental biological characteristics with humans. It is a eukaryote that develops by way of embryogenesis, produces eggs and sperm, exhibits observable behaviors – including the seeking of mates. Its relative simplicity makes it a great subject to study in order to gain insight regarding homologous processes and phenomena in humans. A better understanding of *C. elegans* means better understanding of humans.

C. elegans has several attributes that make it an optimal model organism for laboratory study. One is its short reproductive cycle: it takes only about three days for an egg to develop into an egg-laying adult (Corsi et al., 2015). Also, it is an androdioecious species, meaning that it exists primarily as self-fertilizing hermaphrodites, with males occurring at a low frequency (in the wild type strain N2, males occur at a frequency of 0.1-0.2%) (Corsi et al., 2015). It is therefore easy to generate and maintain genetically homogeneous *C. elegans* populations. This is particularly useful when working with mutant strains. If a higher frequency of males is desired, this can be easily achieved by using *him-8*, a strain that has 37% male frequency because of a higher rate of nondisjunction of the X chromosome (Hodgkin et al., 1979).Additionally, *C. elegans* is easy to grow in a laboratory setting: it can be grown in 6-cm petri plates filled with nematode growth media (NGM) coated with OP50 *E. coli*.

In 1998, *C. elegans* became the first multicellular organism to have its entire genome sequenced (Science, 1998). The availability of this information greatly aids the study of gene function (e.g. in creating mutants). Further, *C. elegans* is eutelic, meaning that the number of somatic cells is constant across all individuals in the species (Sulston et al., 1983). The process and time frame of cell development is constant across individuals as well. Reproducible lineage of every cell's development has been mapped from zygote to adult (Sulston et al., 1983). Plus, since *C. elegans* is transparent, studies can easily be done using a green fluorescent protein (GFP) marker as it is easy to identify the fluorescence in a precise location. GFP can be used in the nematode to visualize certain anatomical structures or functions, such as by labelling individual neurons or observing their activity (Narayan et al., 2016) (Hobert and Loria, 2005). It can also be used as a reporter to determine gene expression (Hobert and Loria, 2005).

Another useful aspect of *C. elegans* that makes it a well-established organism to work with is the fact that its nomenclature (specifically genetic nomenclature) is standardized. Gene names all consist of three or four letters, a hyphen, and an Arabic number (Riddle et al., 1997). For genes defined by mutation, the name is typically indicative of the mutation that was originally found or that is most easily scored (e.g. *him* stands for High Incidence of Males) (Riddle et al., 1997). Genes that have related properties generally have the same three letters and different numbers (e.g. various high incidence of male genes include *him-1, him-3, him-5*, and *him-8*) (Riddle et al., 1997). If the allele is to be denoted as well, this is shown through one or two italicized letters (based on laboratory of isolation) and an Arabic number, in parentheses after the gene name (e.g. *him-8* (v287)) (Riddle et al., 1997). The last element of nomenclature used in this paper is strain. A strain is a group of individuals that possess a certain genotype and are capable of passing that genotype down to progeny. Strains are denoted by two unitalicized, capital letters (also based on laboratory of isolation), followed by Arabic numbers (Riddle et al., 1997) (e.g. CB1489 *him-8(e1489)*).

These attributes of *C. elegans*, along with its with its homology to humans, make it an ideal model organism.

Chemical Sensation in *C. elegans*

Being unable to hear or see, *C. elegans* relies strongly on chemosensation, or the detection of olfactory and gustatory chemicals in the environment (Bargmann, 2006). A significant portion of *C. elegans*' nervous system is devoted to this process. Due to the eutelic nature of the worm, the developmental lineage of every cell has been mapped, resulting in the complete mapping of the physical nervous system (White et al., 1986). Thus every neuron in *C. elegans* is known, including the chemosensory neurons, which regulate chemosensation. The nematodes' olfactory system consists of fourteen pairs of amphid neuron in the head and two phasmid neuron pairs in the tail (Reilly and Srinivasan, 2017). These chemosensory neurons extend dendrites to the tip of the nose or the tail, respectively, where their sensory cilia are exposed to the environment and are able to sense both biotic and abiotic stimuli (reviewed in Bargmann, 2006) (Reilly and Srinivasan, 2017). These cilia can sense hundreds of different chemicals and concentrations due to the expression of several highly localized G protein-coupled receptors, which have high scent specificity (Reilly and Srinivasan, 2017) (Troemel et al., 1995). It is estimated that roughly half of the approximately 1200 GPCRs encoded in the *C. elegans* genome are expressed within the chemosensory neurons (Bargmann, 2006) (Chute and Srinivasan, 2014). Upon receptor activation by an odorant, a signal transduction pathway is initiated, using cyclic GMP (cGMP) as a second messenger to open either TRPV or cGMP-gated ion channels (Bargmann, 2006) (Figure 2). Na^+ and Ca^{2+} flow through these channels, depolarizing the neuron, and the graded potential travels down the nerve to the brain (This differs from human neurons, which use action potentials, an 'all-or-nothing' response). The appropriate behavioral response (e.g. movement towards or away from the stimulus) is then carried out by the animal.

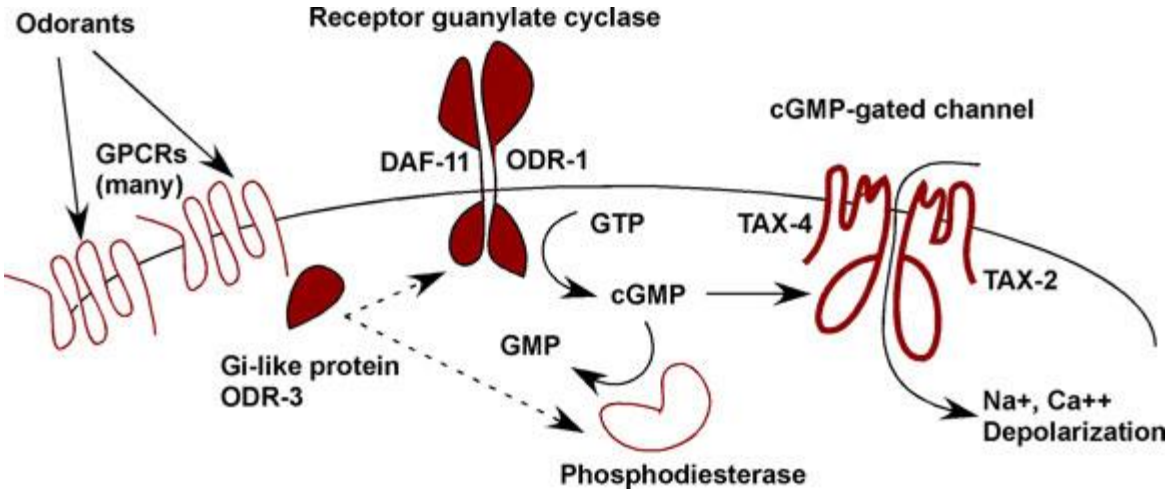


Figure 2: Potential signal transduction pathway of receptor sensing an odorant. (Bargmann, 2006).

C. elegans uses chemosensation to avoid dangerous or nonideal conditions, regulate its development, and to locate food or mates (Bargmann, 2006). In many cases, the chemical signals being sensed are pheromones released by other *C. elegans*. Along with other members of the phylum, *C. elegans* uses a large class of small-molecule pheromones, termed ascarosides, for a variety of communicative purposes.

Ascr#8 is a mating cue

Many pheromones used by *C. elegans* belong to a group of chemicals called ascarosides, which contain an ascarylose sugar, a lipid side chain, and additional building blocks derived from amino acids (Figure 3) (Ludewig and Schroeder, 2013) (von Reuss, et. al, 2012). The term “ascaroside” was originally introduced in 1912 when Flury found a distinct lipid in the parasitic roundworm, *Ascaris lumbricoides* (Ludewig and Schroeder, 2013). In 1982, the first ascaroside in *C. elegans* (known colloquially as Dauer pheromone) was found and was characterized as a “small molecule signal” that regulated dauer formation, or the developmental arrest of larvae in environments with adverse conditions, such as high population density or limited food (Golden and Riddle, 1982). In this alternative form, the larvae are non-feeding and highly stress-resistant. When conditions improve and higher levels of a certain “food pheromone” indicate more adequate resources, the larvae resume regular development (Golden and Riddle, 1982). The chemical composition of dauer pheromone was finally ascertained over 20 years later, with several laboratories contributing findings from 2005-2009, and it was revealed that ascarosides #1, #2, and #3 were the pheromone’s major constituents (Ludewig and Schroeder, 2013). In addition to dauer induction, different ascarosides cause other various responses, such as avoidance, aggregation, or attraction to a mate. For example, octopamine-succinylated ascaroside #9 (osas#9) is a social signaling avoidance pheromone that is produced by individuals under starvation conditions to induce other worms to avoid the area (Artyukhin et. al, 2013). Several ascarosides also induce an attraction response. Indole ascarosides, such as icas#3 and icas#9, strongly attract hermaphrodites and/or males, depending on concentration (Ludewig and Schroeder, 2013). icas#3 can also cause hermaphrodite aggregation on food. In contrast, many non-indole ascarosides cause

hermaphrodite repulsion (and/or male attraction) (Ludewig and Schroeder, 2013). For example, ascr#3 and ascr#8 have been found to attract males. This research project focused on male attraction to ascr#8.

Some ascarosides are species-specific, but as with pheromones in general, others are effective in multiple species. For instance, ascr#9 is utilized by several species of nematode that associate considerably with insects in their natural environment (Choe et al., 2012).

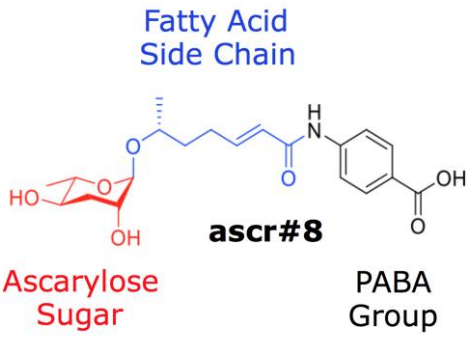


Figure 3: Chemical structure of ascr#8. The molecule consists of an ascarylose sugar (in red), fatty acid side chain (in blue), and a PABA (para-aminobenzoic acid) group (in black).

The pheromone ascr#8 is a mating cue produced by hermaphrodites to attract males and hold them in close proximity (Chute and Srinivasan, 2014) (Simon and Sternberg, 2002). It is composed of the typical ascarylose sugar and unsaturated fatty acid side chain, with its additional building block being a para-aminobenzoic acid (PABA) group (Figure 3) (Pungaliya, 2009). It is a sex-specific pheromone, as it is produced only by hermaphrodites and elicits an attraction response only in males (Chute and Srinivasan, 2014). In a natural environment, once the male reaches the hermaphrodite, it begins tracing the body in search of the vulva so it can begin the mating process. This attractive behavior can also be visualized in the laboratory using behavioral assays, such as the spot retention assay (Figure 4). The optimal concentration of ascr#8 for inducing an attraction response has been found to be 1 µM, as the neurons that sense the pheromone show the greatest response at that concentration (Narayan et al., 2016). Therefore, 1 µM is the concentration of ascr#8 that was used in this project.

In *C. elegans*, ascr#8 is detected by a male-specific class of neurons called cephalic male sensory neurons, abbreviated CEMs. The CEMs include four radially symmetric positioned neurons (as opposed to the bilateral symmetry seen in other neurons): dorsal left, dorsal right, ventral left, and ventral right. They are considered to comprise their own neuronal class because of their locational symmetry, the morphology of their processes and their nuclei, and their gene expression profiles (Narayan et al., 2016). They are regarded as responsible for sensation of certain ascarosides that induce the male attractive response, as it has been suggested and the response characterized by several tests. Particularly, exposure to ascr#8 caused some of the CEMs to depolarize and some others to hyperpolarize, indicating responsiveness. In contrast, exposure to water did not cause a response. Additionally, *C. elegans* males whose CEMs have been removed by laser ablation no longer show the attractive response to ascr#8 (Narayan et al., 2016). The interactive function of the CEMs appears to

cause a concentration-tuned response, with the attraction peaking at a certain ascaroside concentration (1 µM for ascr#8).

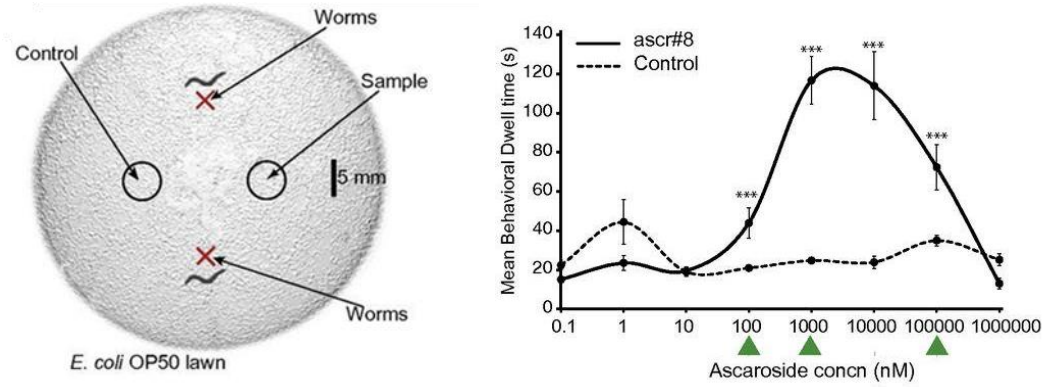


Figure 4: Male *C. elegans* attractive response to ascr#8. (Left) Schematic of Spot Retention Assay, previously used to determine attractiveness of ascaroside. 10 worms were placed on a plate with a drop of control (dH2O) or sample (ascr#8) and mean dwell time of the worm in each was measured. **(Right)** Mean dwell times of worms in ascr#8 (solid line) vs. control (dotted line) at increasing ascaroside concentration shows concentration-dependent attractive response to ascr#8. (Narayan et al. 2016).

C. elegans and related androdioecious species

In nematodes, females (and hermaphrodites, if present in the species) are XX and males are XO. Rather than possessing a Y chromosome like males of most species, male nematodes simply lack a second X, due to an error during meiosis called a non-disjunction (Lui and Colaiácovo, 2013). The hermaphrodites appear (and are) anatomically female, but their first germ cells to differentiate become sperm, and are stored to be used later for self-fertilization. Subsequent germ cells differentiate into oocytes (Ellis and Lin, 2014). Therefore, progeny can be produced through hermaphrodite self-fertilization, or by mating with a male.

Androdioecy evolved independently in three species in the Elegans subgroup of the *Caenorhabditis* genus: *C. elegans*, *C. briggsae*, and *C. tropicalis* (formerly known as *c.sp.11*) (Figure 5) (Felix et al., 2014) (Ellis and Lin, 2014). All events involved in the evolution of hermaphroditism can fall into three categories: (1) new genes were generated via duplication and were recruited to the sex-determination pathway, (2) existing germline genes were independently incorporated into the pathway, or (3) the core pathway itself was modified (Ellis and Lin, 2014). It seems that the events which led to androdioecy in *C. elegans*, *C. tropicalis*, and *C. briggsae* were similar in that they fell into these categories, but the specific genes involved and changes incurred were unique in each species (Ellis and Lin, 2014).

Androdioecy has a few negative implications for the species. For example, males of androdioecious species have reduced fitness compared to those of gonochoristic (male/female) species. Particularly, they are less aggressive (Ellis and Lin, 2014). There is also reduced genetic diversity due to the high frequency of progeny that come from hermaphrodite self-fertilization. The maintenance of genetic diversity is likely the main reason that *C. elegans*

hermaphrodites still produce ascr#8 to attract males despite their ability to reproduce on their own.

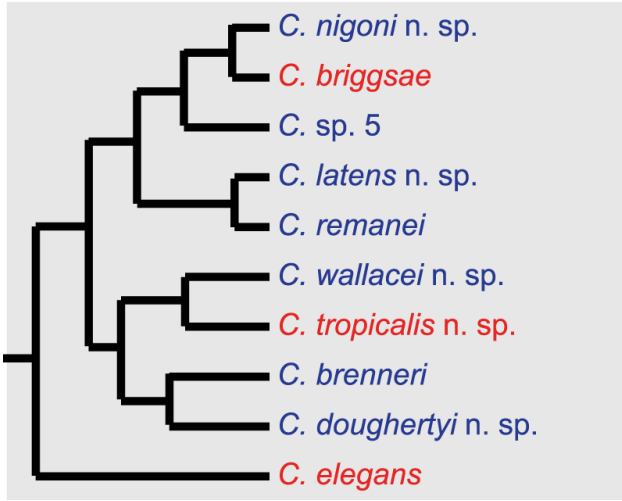


Figure 5: Phylogeny of the *Caenorhabditis* genus. The "Elegans Supergroup". Hermaphroditic species are highlighted in red. It can be seen that each case of hermaphroditism evolved separately. (Felix et al., 2014).

Since *C. elegans* males show an attractive response to ascr#8 secreted by conspecific hermaphrodites, and certain ascarosides have been shown to be effective across closely related species, I performed behavioral assays to investigate if the attractive response was ubiquitous across all hermaphroditic species in the *Caenorhabditis* genus.

Methodology

Worm Strains

The wild type strain of *C. elegans*, N2, originally derived from soil by Sydney Brenner in Bristol, England and introduced to the scientific community in 1973, is used as a control in most behavioral and genetic research of the roundworm (Brenner, 1973). However, N2 was not used as the control in the behavioral assays in this project because the assays required great numbers of males, and N2 has a very low male frequency in populations (0.1-0.2%) (Corsi et al., 2015). The *him-8* strain, however, is a control strain that has a higher frequency of males (37%) due to an increased rate of nondisjunction of the X chromosome (Hodgkin et al., 1979). Therefore, CB1489 (*him-8*(e1489)) was obtained from the *Caenorhabditis* Genetics Center (CGC) and was used as the control for behavioral testing.

The second experimental strain used was *C. tropicalis him-8* (RE1017(*ctr-him-8*(v287))). The wild type isolate of *C. tropicalis* (JU1373) is hermaphroditic, so RE1017 was created as a *him-8* mutant in the wild type background by Ronald Ellis. Formerly known as *C.sp.11* or "species 11", *C. tropicalis* is a species related to *C. elegans* (Figure 5) (Felix et al., 2014). The *him-8* (v287) mutant has a similarly high male frequency to the *C. elegans* homolog, resulting in 30-35% males, and was obtained from Dr. Ronald Ellis at Rowan University.

The third experimental strain used was *C. briggsae him-8* (RE980(*cbr-him-8*(*v188*))). This is the third androdioecious species in the *Elegans* subgroup of the *Caenorhabditis* species (Ellis and Lin, 2014). This strain also was a *him-8* mutant of the wild strain AF16 and was obtained from Dr. Ronald Ellis.

Worm Maintenance

Agar Plates

All three nematode species were maintained on 60 mm standard nematode growth media (NGM) agar plates seeded with OP50 strain *E. coli* as a food source. OP50 was used because its slow growth on NGM agar allows for clear observation of the worms. 1 liter of NGM was made by combining 55mM NaCl, 0.25% Peptone, 1.7% Agar, 5 mM Cholesterol, 1 mM MgSO4, and 2.5 mM KPO4 as described in Wormbook (Stiernagle, 2006). The solution was aseptically dispensed into plates. Plates were left to dry at room temperature for 2-3 days to allow for detection of any contaminants and to allow evaporation of excess moisture (Stiernagle, 2006). OP50 (a few drops) was then added to the plates via pipet, using sterile technique.

C. elegans Maintenance

Every 2-4 days, approximately five young adult worms were passed onto a fresh plate to prevent overcrowding. Passing was performed using a platinum wire pick, sterilized by heating to red hot in the flame of an ethanol lamp between each worm. The worms were also sometimes passed by means of “chunking,” in which a small section of agar from a well-populated plate was cut with a metal spatula (dipped in ethanol and sterilized in flame) and placed face down onto a new seeded plate. Plates were maintained in a 20 °C incubator.

C. tropicalis Maintenance

C. tropicalis was maintained in a similar way to *C. elegans*. However, due to differences in the natural habitat of the species and optimal growth temperature, *C. tropicalis* plates were kept in a 25 °C incubator.

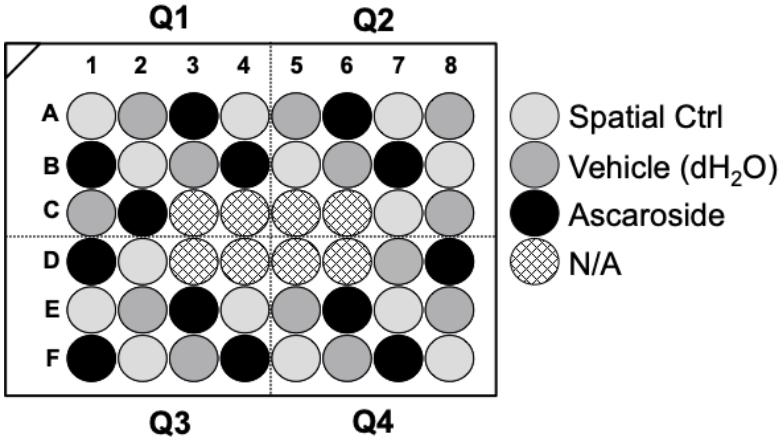
C. briggsae Maintenance

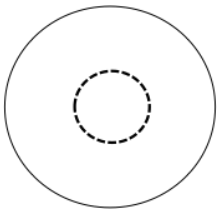
C. briggsae was maintained similarly overall to the other two strains. However, it was observed that several to all individuals on a plate tended to crawl off the agar onto the plate wall, where they would dry up and die. It was presumed (but uncertain) that this was due to greater sensitivity to overcrowding. To best work around this problem, double the typical number of plates were passed for maintenance, to maximize the odds of having a plate that grew successfully. Also, some plates of *C. briggsae* were maintained at 20 °C while others were

maintained at 25 °C due to uncertainty of optimal temperature. Because of time constraints of the project, some assays were performed with individuals from both temperatures.

The Single Worm Behavioral Assay (SWA)

L4 and young adult males were isolated from hermaphrodites 5-16 hours prior to assay, as described previously (Narayan et al, 2016). Forty to fifty worms were passed onto a plate seeded with OP50 and were maintained at the maintenance temperature for each species until the time of the assay.





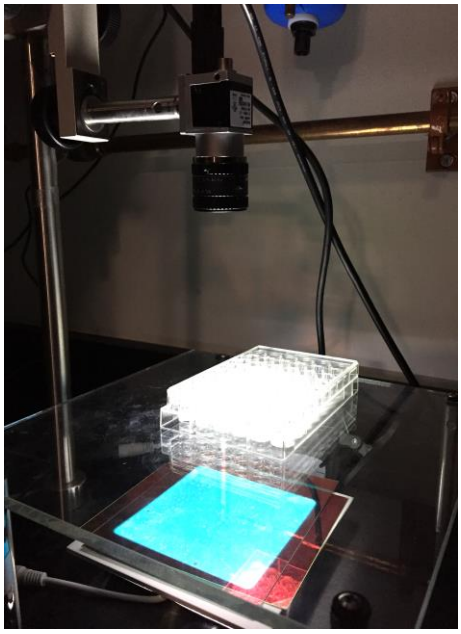


Figure 6: Single Worm Assay Setup (Top Left) 48-well tissue culture plate seeded with 200 µL Nematode Growth Medium and 65 µL OP50 *E. coli* media. One quadrant at a time, 0.85 µL of either vehicle or ascr#8 was added to appropriate wells, and plate was recorded in WormLab. 4 quadrants were analyzed to give n=1. **(Top Right)** Close up of example well with approximate center size outlined. **(Bottom)** Assay plate on camera stage, lit from below, during recording.

The assay utilizes a 48-well tissue culture plate seeded with 200 µL NGM in all but eight of the wells (Figure 6). The empty wells in the center ("N/A" wells in Figure 6) were not used because their required drying time far exceeds that of other wells and leads to outer wells drying to the point of being unusable. This plate was seeded using a multichannel pipette to transfer 0.85 µL of OP50 *E. coli* media onto the NGM-containing wells. The plate was then dried in a warm room (37 °C) for 3.5 hours with the lid left open. If a plate was not used immediately, it was wrapped in Parafilm to prevent it from drying out.

For each experiment, three well conditions were designated and used: Spatial control (S) wells had nothing additional added to the well center, and were used to assess how much time nematodes spend in the middle just by chance. Vehicle (V) wells had 0.85 µL dH2O added to the center to evaluate how attractive liquid in general was to the worms. Ascaroside (A) wells had 0.85 µL ascr#8 added to the center in order to test the worms' level of attraction to the pheromone.

At the time of the assay, PCR tubes of water and 1 µM ascr#8 were thawed on ice. 0.85 µL of water or ascr#8 was pipetted into the center of appropriate wells in the first quadrant of the 48-well plate, as shown in Figure 6. Spatial control wells were left empty accordingly. A single worm from the isolation plate was then passed onto each well in the quadrant. The plate was placed on a camera stage. The camera iris was adjusted for optimal visibility of the worms in the video, and video was then recorded for 15 minutes using the nematode-tracking program, WormLab (WormLab from MBF Bioscience). Pipetting of water and ascr#8, passing of worms onto wells, and recording was then repeated individually for each of the quadrants on the plate.

Once completed, the video recordings were manually analyzed to track time spent by each worm in the middle of its well. This analysis was performed as follows:

Data Analysis

Raw Analysis

Focusing on one well at a time, the entire length of the video was examined to observe the times in which the worm was in the well center. On a per worm basis for each quadrant of the plate, the time in and time out for each visit were recorded in Microsoft Excel. A visit was defined as a period of at least ten elapsed seconds between the moment the worm's nose entered the center ("time in") and the moment its tail exited ("time out"). In V and A wells, the boundaries of the center were set by the outlines of the water or ascaroside drop, if visible. In S wells, along with V or A wells in which the drop lacked a visible outline, the drop size in surrounding wells was used for reference. If there were fewer than ten seconds between two visits, they were combined and counted as one. Number of visits and the mean duration of a visit (i.e. mean dwell time) were calculated.

Determination and Removal of Outliers

Wells with mean dwell time values greater than three standard deviations above the mean for each condition were deemed outliers, and were excluded from the following data sets.

Calculating Percent Attractiveness

The following was performed for the vehicle and ascaroside conditions. Any visit greater than 2 standard deviations above the mean vehicle dwell time for plate was counted as an attractive visit, and was scored as “1”. Any that were not attractive were scored as “0”. The percentage of attractive visits in each well was then calculated, and these percentages for were averaged for the each quadrant. The mean of the quadrant averages was then obtained to yield the percent attractiveness for the whole plate (one value for vehicle, and one value for ascaroside).

Statistical Analyses

Normality Check

A Shapiro-Wilk Normality test, which allows assessment of normality using an $n < 10$, was utilized to validate the use of parametric statistical analyses.

Internal Strain Comparison

A Repeated Measures One-Way Analysis of Variance (RM-ANOVA) was performed to compare the vehicle value to the spatial and to the ascaroside. If a comparison yielded $p < 0.05$, a Bonferroni’s test was performed when making multiple comparisons.

Spatial Control Check

An ANOVA was also run to compare the mean dwell times of spatial controls in each species, to ensure that they were not significantly different. By making sure the spatial controls are the same, this ensures that any differences observed in experimental conditions are not due to differences in locomotion. A Dunnett’s test was performed when making multiple comparisons.

Transformation by Log(Fold-Change)

To be able to compare data across species, the data was normalized by taking log(fold-change) of the increase from vehicle to ascaroside. To do this, the fold-change was first determined by dividing the average dwell time in ascaroside by the vehicle for each plate. Because this alone led to values ranging from <1 to 90, the log of each value was then calculated, so that the range would be confined to -1 to 1. This also had the side effect of normalizing the data. An ANOVA was then performed, followed by a Bonferroni’s test to see if the values differed between species.

Results & Discussion

Although the *him-8* strain was used rather than the wild-type N2, *him-8* behaviors the are same as N2. The only different is the rate of non-disjunction during meiosis. Therefore, the data gathered from this research is applicable to make conclusions about the response of N2 worms to ascr#8.

The first metric taken was mean dwell time, or the average amount of time that the worms spent in the well center. Dwell time was calculated as Time in – Time out for each visit,

and the mean values for each plate were averaged to yield mean dwell time for the species. Mean dwell time data can be seen below in Figure 7.

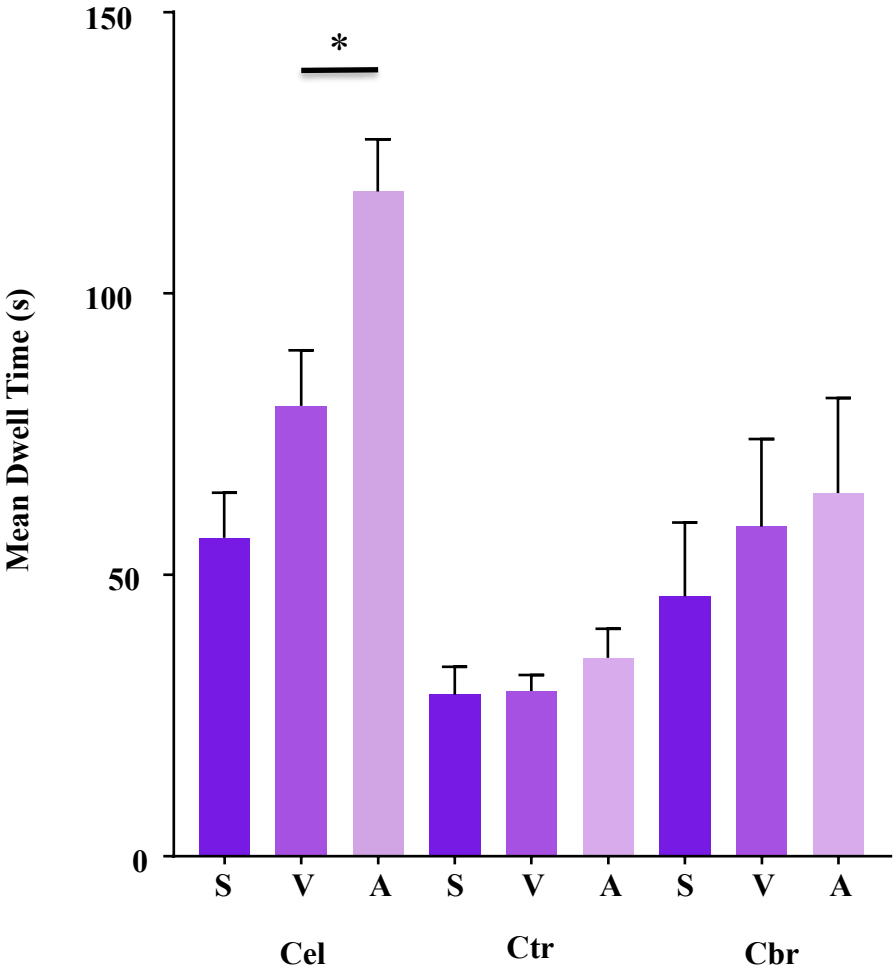


Figure 7: Mean Dwell Time. *C. elegans* showed a significant attractive response to ascr#8 (p=0.0052 by RM-ANOVA), while neither *C. tropicalis* nor *C. briggsae* did.

As expected based on previous literature (Pungaliya, 2009) (Narayan et al., 2016), *C. elegans* males showed an attractive response to ascr#8. This is seen by the fact that the species' ascaroside mean dwell time was significantly higher than its vehicle mean dwell time. This confirms that the assay worked and serves as a proof-of-principle that males of the species are attracted to the pheromone. In contrast to *C. elegans*, neither *C. tropicalis* nor *C. briggsae* showed an attractive response to ascr#8.

Although it is seen that *C. elegans* had the greatest difference between mean dwell times for vehicle and ascaroside, the data in Figure 7 is not optimized for making comparisons between species because the values for each are so different. Therefore, in order to normalize the data and be able to properly make interspecies comparisons, the fold-change was calcuated

by dividing ascaroside dwell time by vehicle dwell time (Figure 8). This normalized the A-to-V data as ratios, and were then further normalized by calculating the log of these values. Log(fold-change) data is displayed in Figure 8.

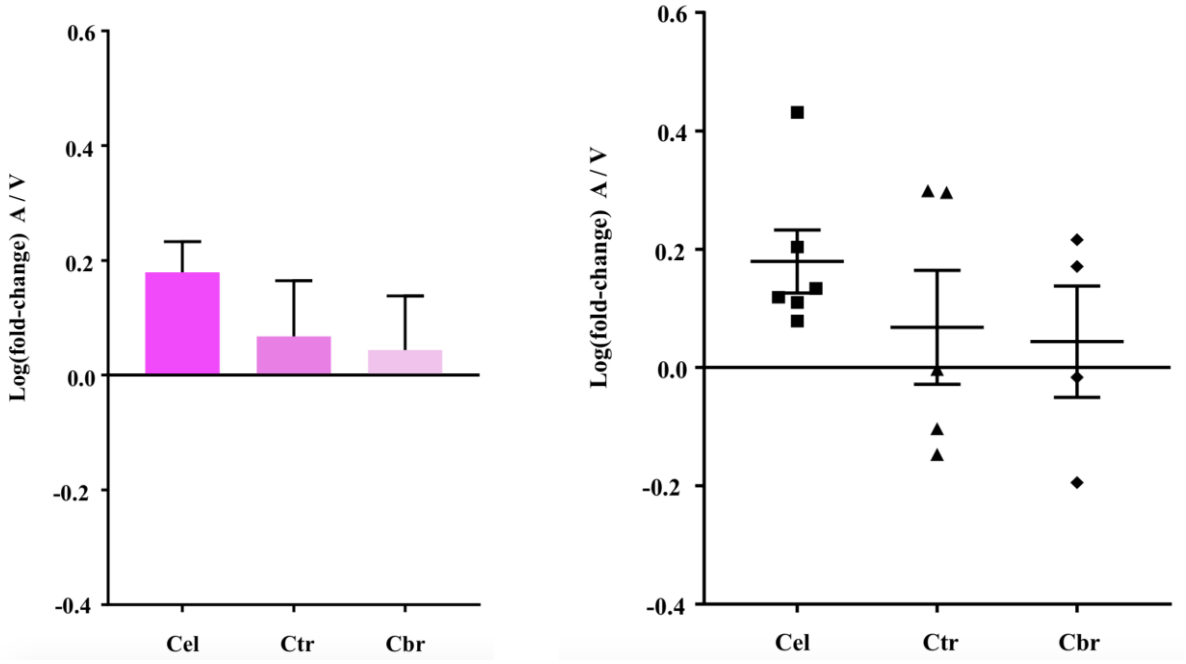


Figure 8: Log(Fold-Change). (Left) *C. elegans* showed the greatest increase in dwell time from A to V. *C. tropicalis* and *C.* briggsae on average showed a slight increase. **(Right)**However, examining the values for individual plates shows that *C. elegans* strongly clusters in the positive, *C. tropicalis* and *C. briggsae* data points are distributed differently across the x-axis.

C. elegans has the most positive log(fold-change), indicating that it had the greatest increase in dwell time from vehicle to ascaroside among the three species. Examining the values for individual plates shows that *C. tropicalis and C. briggsae* on average show a slight increase, but upon examination of the individual data points (Figure 8, right) it is revealed that the data is distributed differently than that for *C. elegans*. The values for both species varied from negative, to nearly 0, to positive. It appears that the extreme positive values drive up the average log(fold-change), so the average does not tell the whole story.

It is even possible that *C. tropicalis* would show a bimodal distribution if more plates were run, which would be a great difference from *C. elegans*. If that is the case, it could have several possible explanations: Perhaps some individuals are able to perceive ascr#8, while others are not. Alternatively, perhaps all individuals of *C. tropicalis* can perceive the pheromone but only some show an attractive response to it.

Another metric taken was the visit count, or average number of visits of each species in each condition. This data is below in Figure 9.

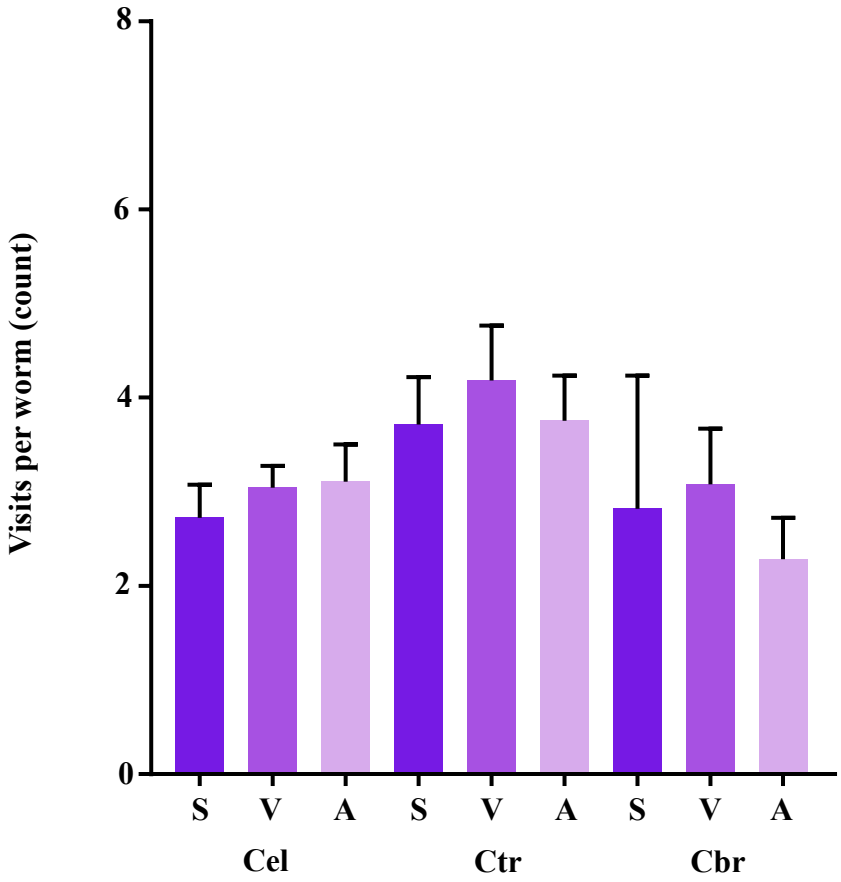


Figure 9: Visit Count Per Worm. No significant differences were seen between species.

There was not a significant difference in the visit counts within or between species. This strengthens the validity of the data in Figure 7, as it shows that higher dwell time of *C. elegans* in ascr#8 is not due to more visits. Similarly, lower dwell time in *C. tropicalis* (and *C. briggsae*) is not due to fewer visits.

Percent attractiveness was also calculated for vehicle and ascaroside in each species, and was defined as the percentage of visits with a dwell time greater than two standard deviations above the mean of the vehicle. The values for percent attractiveness are in Figure 10.

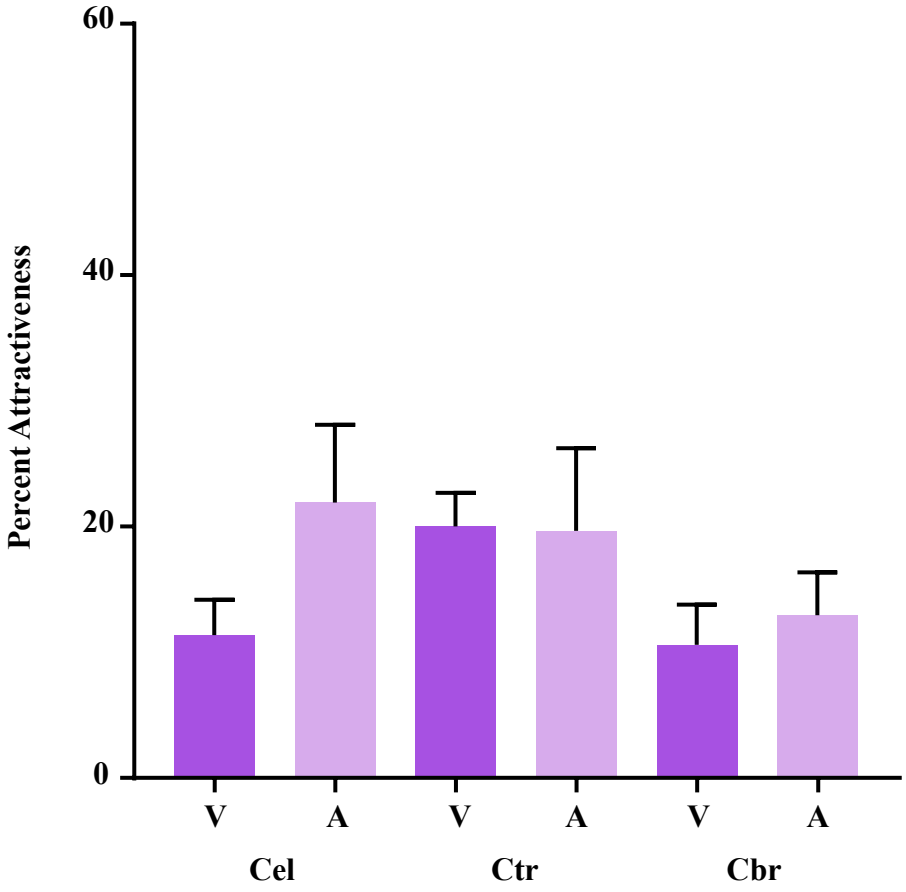


Figure 10: Percent Attractiveness. There was not a significant difference in percent attractiveness to vehicle vs. ascaroside in any species.

There was not a significant difference between the percent attractiveness to vehicle and to ascaroside in any of the species. However, in *C. elegans*, the difference between attractiveness to ascaroside and to vehicle trends higher ($p = 0.0757$ by ANOVA), while in *C. tropicalis* and *C. briggsae*, the values for vehicle and ascaroside are more similar to each other.

Similarly to how log(fold-change) was used to normalize the mean dwell time data, log(fold-change) was also used to normalize percent attractiveness data. The results are seen below in Figure 11.

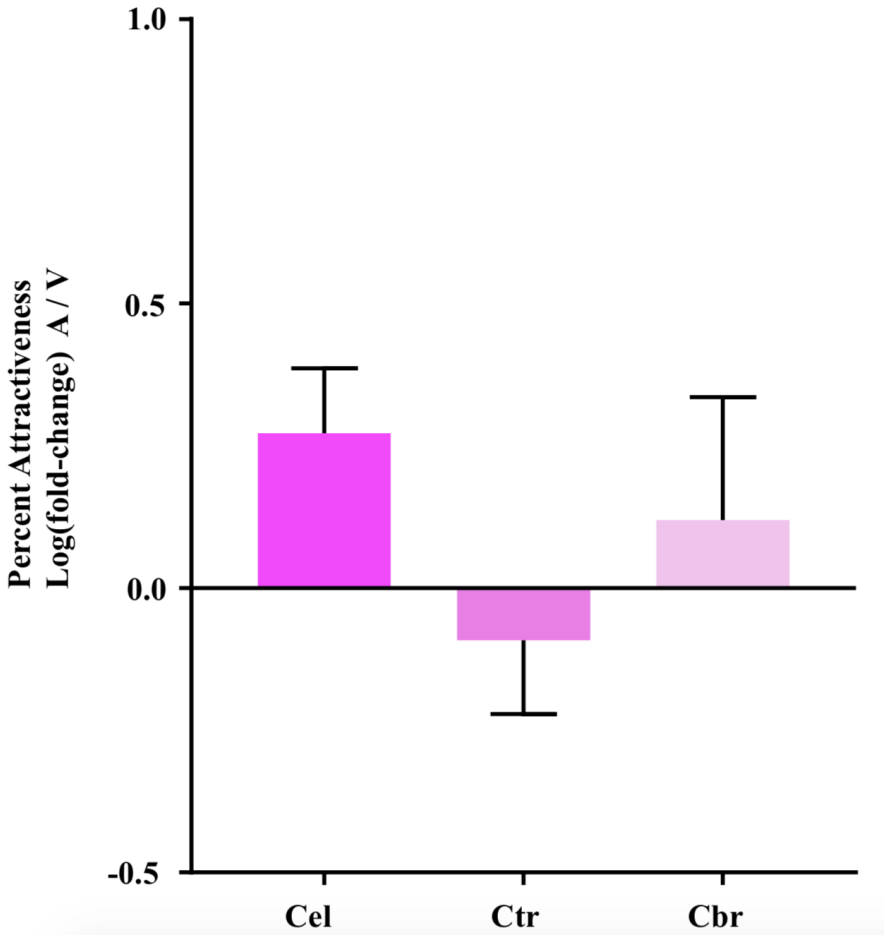


Figure 11: Log(Fold-change) of Percent Attractiveness. *C. elegans* has the most positive log(fold-change) of percent attractiveness of the three species, indicating increase attractiveness from vehicle to ascaroside. *C. tropicalis* has a slightly negative value, indicating a decrease. *C. briggsae* is slightly positive.

C. elegans shows the most positive log(fold-change) of percent attractiveness from ascaroside to vehicle, indicating that the increase in attractive visits seen from ascaroside to vehicle was greatest in *C. elegans* males. *C. briggsae* also has a positive value, signifying a smaller increase. In contrast, *C. tropicalis* has a negative value, meaning that the percent attractiveness to ascaroside was actually less than that to the vehicle. It is possible that this indicates avoidance, as past research in the lab has found a correlation between negative log(fold-change) A/V values and avoidance of the chemical being tested.

Also worth noting is that the mean dwell times of *C. tropicalis* were the lowest of all the species tested, for each condition, so it could also be possible that the worms prefer to be dry rather than wet. If avoidance were the case, then the response would be mild, as the value is not strongly negative. The possibility of an avoidance response could be tested using more single worm assays, along with and avoidance assays.

Additionally, the number of tests run for each species was as follows: *C. briggsae* only had n=4 due to time constraints, *C. tropicalis* had n=5, and *C. elegans* had an n=6. It is possible that a greater n, such as n=10, may better show differences between the species.

Conclusions & Future Research

The research suggests that the attractive response of males to ascr#8 is unique to *C. elegans*. This may be related to the fact that hermaphroditism arose independently in the three tested species. Since the three species evolved separately (on different branches of the phylogenetic tree of the genus), they each went through the process of natural selection differently, and this is likely seen in many of their characteristics. So, it would make sense that their methods of attracting mates would be different.

More remains to be explored about ascr#8 and its function in the nematodes. In particular, future research could investigate whether the pheromone has effects other than inducing a male attractive response. For example, perhaps hermaphrodites show an avoidance response because the presence of ascr#8 indicates that another hermaphrodite is in the vicinity and would be a source of competition for attracting mates. Additionally, perhaps ascr#8 can induce a developmental response such as dauer formation. Ascarosides can serve several different functions, so it is not unlikely that this applies to ascr#8 as well. Conducting research to further explore the effects of ascr#8 would lend to a fuller characterization of the pheromone and what pheromones are capable of in general.

More potential related research could look into mate-attracting strategies in *C. tropicalis* and *C. briggsae*. Since they are not attracted to ascr#8, they likely have their own attractive pheromones since having a method of attracting a mate of one's own species is rather crucial for species continuation and evolutionary fitness. Identifying such mechanisms in these species this would provide a more complete picture of the evolution of the different species and also of brain differences between sexes.

Overall, this research can be taken further in several different directions. There is much to be learned about *C. elegans* and ascr#8, along with *C*. *tropicalis* and *C. briggsae* and their own modes of mate attraction. This research fits under the larger scope of how different species evolve, which underpins all life. Better understanding pheromone signaling in nematodes can ultimately give insight into neuronal function and its influence on behavior that is expandable to countless other animals, even humans.

Acknowledgments

I would like to thank Dr. Jagan Srinivasan, Douglas Reilly, Elizabeth DiLoreto, and the rest of the lab for their guidance and support throughout the course of this project. I would also like to thank Dr. Ronald Ellis at Rowan University for providing the *C. tropicalis* and *C. briggsae* strains to the lab. *C. elegans* was obtained from the *Caenorhabditis* Genetics Center (CGC).

References

Artyukhin, A.B., Schroeder, F.C., and Avery, L. (September 27, 2013). Density dependence in *Caenorhabditis* larval starvation. *Scientific Reports*. Retrieved from https://www.nature.com/articles/srep02777

Bargmann, C.I. (October 25, 2006). Chemosensation in C. elegans, WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.123.1, http://www.wormbook.org.

Brenner, S. (December 10, 1973). The genetics of *Caenorhabditis elegans*. *Genetics*. Retrieved from http://www.genetics.org/content/genetics/77/1/71.full.pdf (Brenner, 1973)

Choe, A., von Reuss, S. H., Kogan, D., Gasser, R.B., Platzer, E.G., Schroeder, F.C., Sternberg, P.W. (2012). Ascaroside signaling is widely conserved among nematodes. *Current Biology, 22*(9), R294-R296. https://doi.org/10.1016/j.cub.2012.03.024

Chute, C.D. and Srinivasan, J. (2014) Chemical mating cues in C. elegans. *Seminars in Cell & Developmental Biology, 33*, 18-24. doi: 10.1016/j.semcdb.2014.06.002

Corsi A.K., Wightman B., and Chalfie M., (June 18, 2015). A Transparent window into biology: A primer on *Caenorhabditis elegans*. *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.177.1, http://www.wormbook.org.

Ellis, R.E. and Lin, S.Y. (2014). The evolutionary origins and consequences of self-fertility in nematodes. *F1000prime reports, 6*, 62. doi:10.12703/P6-62

Félix, M. A., Braendle, C., & Cutter, A. D. (2014). A streamlined system for species diagnosis in Caenorhabditis (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. *PloS one*, *9*(4), e94723. doi:10.1371/journal.pone.0094723

Golden, J.W. and Riddle, D.L. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. *Science*, *218*(4572), 578-580. doi:10.1126/science.6896933

Hobert, O. and Loria, P. (2005). Uses of GFP in *Caenorhabditis Elegans* . In Green Fluorescent Protein (eds M. Chalfie and S. R. Kain). doi:10.1002/0471739499.ch10

Hodgkin, J., Horvitz, H.R., and Brenner, S. (1979). Nondisjunction mutants of the nematode CAENORHABDITIS ELEGANS. *Genetics*, *91*(1), 67-94. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17248881

Karlson, P. and Lüscher, M. (1959). 'Pheromones': a new term for a class of biologically active substances. *Nature*. Retrieved from https://www.nature.com/articles/183055a0.pdf (Karlson, Luscher 1959)

Ludewig A.H. and Schroeder F.C. (January 18, 2013) Ascaroside signaling in *C. elegans*, *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.155.1, http://www.wormbook.org.

Lui, D. Y., & Colaiácovo, M. P. (2013). Meiotic development in Caenorhabditis elegans. *Advances in experimental medicine and biology*, *757*, 133–170. doi:10.1007/978-1-4614-4015-4_6

Michigan State University. (2008). *Animal Communication: Introduction and Evolutionary History.* [PowerPoint slides]. Retrieved from https://msu.edu/course/zol/313/summer2008/Lectures/Communication1.ppt

Narayan, A., Venkatachalam, V., Durak, O., Reilly, D.K., Bose, N., Schroeder, F.C., Samuel, A.D.T., Srinivasan, J., Sternberg, P.W. (March 8, 2016). Contrasting responses within a single neuron class enable sex-specific attraction in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(10), E1392-E1401. https://doi.org/10.1073/pnas.1600786113

Pungaliya, C., Srinivasan, J., Fox, B. W., Malik, R. U., Ludewig, A. H., Sternberg, P. W., & Schroeder, F. C. (2009). A shortcut to identifying small molecule signals that regulate behavior and development in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(19), 7708–7713. doi:10.1073/pnas.0811918106

Puyana, M. (n.d.). Chemical signals in coral reefs. *Encyclopedia of Life Support Systems* [Web]. Retrieved from http://www.eolss.net/sample-chapters/c06/e6-52-04-04.pdf

Regnier, F.E. and Law, J.H. (1968). Insect pheromones. *Journal of Lipid Research, 9*(5), 541-551. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/4882034

Reilly, D.K. and Srinivasan, J. (2017). *Caenorhabditis elegans* olfaction. *Oxford Research Encyclopedia of Neuroscience*. doi: 10.1093/acrefore/9780190264086.013.191

Riddle D.L., Blumenthal T., Meyer B.J., et al., editors (1997). C. elegans II. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Part A, Genetic Nomenclature. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK20203/

Sandler B.H., Nikonova L., Leal W.S., Clardy J. (2000). Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. *Chemistry & Biology, 7*(2),143-151. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10662696

Science. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science, 282*(5396), 2012-2018. doi: 10.1126/science.282.5396.2012

Shorey, H.H. (1973) Behavioral responses to insect pheromones. *Annual Review of Entomology, 18*, 349-380. https://doi.org/10.1146/annurev.en.18.010173.002025

Simon, J.M. and Sternberg, P.W. (2002). Evidence of a mate-finding cue in the hermaphrodite nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America, 99*(3), 1598-1603. https://doi.org/10.1073/pnas.032225799

Stiernagle, T. (2006). Maintenance of *C. elegans*, *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.101.1, http://www.wormbook.org.

Sulston, J.E., Schierenberg, E., White, J.G., Thomson, J.N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology, 100*, 64-119. Retrieved from http://hobertlab.org/wp-content/uploads/2013/03/Sulston_embryonic_lineage_1983.pdf

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans, Cell*, 83. Retrieved from https://core.ac.uk/download/pdf/82239971.pdf

University of Minnesota. (n.d.) What is C. elegans? University of Minnesota College of Biological Sciences. Retrieved from https://cbs.umn.edu/cgc/what-c-elegans

Universiteit Utrecht. (n.d.). *The nematode* C. elegans. [Photograph]. Retrieved from https://web.science.uu.nl/developmentalbiology/boxem/elegans_intro.html

von Reuss, S. H., Bose, N., Srinivasan, J., Yim, J. J., Judkins, J. C., Sternberg, P. W., & Schroeder, F. C. (2012). Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in C. elegans. *Journal of the American Chemical Society, 134*(3), 1817–1824. doi:10.1021/ja210202y

Wedekind C., Seebeck T., Bettens F., Paepke A.J. (June 22 1995). MHC-dependent mate preferences in humans. *Proceedings of the Royal Society B,* 260(1359). Retrieved from https://royalsocietypublishing.org/doi/10.1098/rspb.1995.0087

White, J. G. et al. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. [Abstract]. *Philosophical Transactions of the Royal Society B: Biological Sciences, 314*(1165), 1-340. doi:10.1098/rstb.1986.0056

WormLab 2019.1.1 (MBF Bioscience, Williston, T, USA)

Wyatt, T. (2015). How animals communicate via pheromones. *American Scientist, 103,* 114. doi: 10.1511/2015.113.114