Pathogen Avoidance by Caenorhabditis Elegans Is a Pheromone-Mediated Collective Behavior

Pathogen avoidance by *Caenorhabditis elegans* is a pheromone-mediated collective behavior

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Abstract

A model organism *Caenorhabditis elegans* is a free-living nematode that feeds on bacteria in the wild. To survive, it has to be able to distinguish between benign and pathogenic microorganisms. Thanks to its simple neural system it is capable of determining whether the source of food is dangerous and physically avoid it.

In this work we present evidence that this avoidance behavior depends on the nematode population density. The worms exposed to a pathogen secrete an alarm pheromone which drives the population away from the danger. Worms carrying a mutation in the *acox-1* gene (involved in the pheromone biosynthesis) are unable to produce the alarm signal and *odr-7* mutants fail to react to it suggesting that AWA neurons are involved in the pheromone detection. We developed a simple phenomenological model that quantifies the effects of the secreted pheromone and was able to reproduce major features of the nematodes' behavior at the population level.

We show that the collective avoidance is not restricted to *C. elegans* as other *Rhabditida* nematodes also exhibit this behavior. Furthermore, the alarm signal left by *C. elegans* in the bacterial lawn is detected by most of the other species tested but some of them were attracted to it rather than repulsed which suggests that the chemical signal is not conserved between them.

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1

Introduction

Collective phenomena are common in biology at all scales from self-organization of macromolecules[59] to flocking birds[12]. Animal behavior is a particular example that emerges from simple interactions among constituents and in response to changes in the environment. Unlike many other phenomena in biology (e.g. biochemical networks or developing tissues), where it is not clear what the relevant scales are, here the small scale - the individual animal - and the large scale - the group - are easily identifiable and are well separated. A wide range of animal systems has been studied both theoretically

and experimentally over the past 20 years[207]. However, what behavioral studies have in terms of accessibility, phenomenology, and mathematical rigor, they often lack in terms of experimental precision, control over properties of the environment and the interactions, and ability to carry out precise perturbations. Also missing is a closer connection to molecular, genetic and neuronal mechanisms that drive collective behavior. Here, we propose that the organism *Caenorhabditis elegans* can be established as a model for collective behavior that will enable an unprecedented level of control of its environment and access to molecular biology underpinning its interactions between conspecifics.

We investigate bacterial avoidance behavior in which *C. elegans* reverse their initial attraction to a pathogen. In Chapter 2 we will develop an experimental system capable of quantitatively tracking the positions of worms feeding on noxious bacteria and show evidence, that this behavior depends on indirect interactions between the nematodes. Chapter 3 will further quantify this phenomenon using two phenomenological models. In Chapter 4 we will investigate molecular basis of the behavior at the level of neuronal, endocrine and immune systems. Finally in Chapter 5 we will ask whether collective avoidance is restricted only to *C. elegans* or is it more commonly found amongst other nematodes?

1.1 Why do we study *C. elegans* ?

A round worm *Caenorhabditis elegans* was first isolated and described at the turn of the 20^{th} century by Emilie Maupas[143] but it was not until Sydney Brenner started working with it[23] half a century ago, when it became one of the model organisms in biology. This small (1mm in length) free-living non-parasitic hermaphroditic nematode can be easily cultivated in a laboratory where it feeds on benign *E. coli* bacteria. Its short reproductive cycle (3 days) and the fact that each hermaphrodite can lay up to 300 eggs enables researchers to grow large clonal populations in a short span of time. Furthermore, the transparent nature of the organism lent itself to easy observation.

This ease of manipulation of this organism has led to major breakthroughs in biology. In 1983 a complete developmental lineage of all 959 cells has been determined and it turned out that this complicated process was invariant between conspecifics[206]. Shortly after that, a complete map of all neurons and their connections has been determined by a series of cross sectional micrographs[38,2,157,4,233,86,96]. Until now it still remains the only organism whose complete connectome is known. Finally, in 1998 *C. elegans* was the first animal whose genome was sequenced[217] which laid groundwork for completing the Human Genome Project five years later and revealed that 36% of genes of *C. elegans* were homologous to human's.

Now, researchers study almost every aspect of *C. elegans* : from molecular and developmental biology to behavior and evolution and the open source spirit of the scientific community (expressed through open and collaborative projects like WormBook[32], WormAtlas[7] and WormBase[92]) made the nematode easily accessible to non-biologists as a research tool.

1.2 Pathogen detection and avoidance

C. elegans hermaphrodite has 302 neurons that control various muscle contractions, integrate environmental stimuli and act as sensors in the interface between the animal and its environment[13]. Despite the simplicity of *C. elegans* neural system, the nematode is capable of a wide array of complex behaviors. For example, the worm is able to discriminate between nutritious bacterial food and lethal pathogens which is crucial for its survival in the microorganism-rich environment that it occupies[148].

Upon exposure to some novel pathogenic bacteria, *C. elegans* is initially attracted (Fig. 1.1a) to the microorganism (presumably because it is the only source of food in these laboratory conditions), but after several hours it starts to actively avoid it (Fig. 1.1b). The nematodes were hypothesized to detect a particular chemical compound associated with the pathogen and use it to evade the danger.

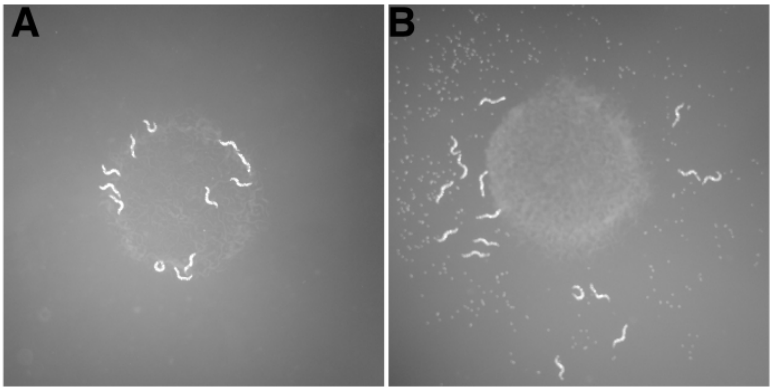

Figure 1.1: *C. elegans* nematodes (bright wiggles) feeding on PA14 bacteria (faint circle in the middle) in a standard avoidance experiment. (A) shows early stages of the experiment when worms are attracted to the pathogen and spend most of the time in the bacteria. (B) shows later stages of the experiment when worms avoid the bacteria.

One can think of pathogen avoidance as the first line of defense in the nematode's immunity helping it save energy involved in production of expensive anti-virulence factors.

This phenomenon was first observed in *C. elegans* -*S. marcescens* system[165] where it was subsequently found that the nematodes avoid a particular biosurfactant (cyclic lipodepsipentapeptide serrawettin W2) produced by the bacteria[163]. The chemical compound was recognized by the worms through AWB olfactory neuron.

In another example *C. elegans* , that are usually attracted to acylated homoserine lactones (AHSL) autoinducers, were shown to avoid them following a preexposure to *P. aeruginosa* PAO1 bacteria[18]. AHSL autoinducers mediate quorum sensing that controls production of virulence factors of PAO1 which in normal conditions is lethal to the worms. The nematodes in these assays were able to associate AHSL autoinducers with the threat.

More recently, it was shown that another strain of *Pseudomonas* - PA14 produces two secondary metabolites (phenazine-1-Carboxamide and pyochelin) that upregulate expression of DAF-7 ligand[174] in ASJ sensory neurons of *C. elegans* feeding on the bacteria. This in turn represses DAF-3 in another set of neurons RIM/RIC and promotes the nematode leaving the bacterial lawn[147]. The

discovery of this pathway was particularly interesting because it relates pathogen avoidance to an array of other behaviors[83] that daf-7 is responsible for such as oxygen sensation[34], aggregation[218] and pheromone detection[186].

These are three examples of pathogen-associated molecular pattern[189] (PAMP) strategy where a cellular receptor recognizes a specific microbial peptide or a toxin (in these cases serrawettin W2, AHSL autoinducers or the two phenazines) and then leads to a behavioral or immune response. This response could be pathogen specific but it will not allow *C. elegans* to mount a defense against a wider range of invaders.

An alternative pathogen detection strategy may also be involved in *C. elegans -P. aeruginosa* PA14 system. This opportunistic pathogen is able to penetrate the worm's intestine intact and inactivate nematode's core cellular processes via the endocytosed translation inhibitor Exotoxin A[56] which leads to upregulation of some immune pathways. This mechanism is at work even in the absence of a pathogen itself as an analogous inactivation of worm's various cellular processes (transcription, translation, secretion etc.) through RNAi expressing *E.coli* strain[149], that is otherwise innocuous, has led to pathogen avoidance behavior similar to one exhibited in case of PA14 strain. This infection response strategy is a damage-associated molecular pattern (DAMP) and in principle can be effective in case of broad range of attackers especially ones that the nematode had not encountered before or did not evolve more specific mechanisms to deal with them.

1.3 Aversive learning

Another case in which *C. elegans* changes its naive behavior upon preexposure to a pathogen is known as aversive learning[245]. In these experiments *C. elegans* are placed on a plate in between two different bacterial lawns located at the opposite sides of the dish (in contrast to avoidance experiments described in the previous section where pathogenic bacteria were the only source of

food). The nematodes are then able to move toward one or the other sources of food depending on their preference. When given a choice of *E. coli* OP50 (standard laboratory *C. elegans* food) and a pathogen *P. aeruginosa* PA14, the worms have a slight preference for PA14. However, if the animals spend several hours on PA14 immediately before the choice assay their preference changes markedly and trained nematodes move toward OP50 food.

This simple assay could be used to elucidate the role of particular neurons and connections in aversive learning behavior. To find whether a particular neuron is necessary in the process one could use a genetic knockout animal [23] with a mutation in a gene responsible for a differentiation of the cell. However, mutants like this exist only for a limited number of neurons, so to knockout the remaining ones one has to resort to laser ablation [15,68]. In this technique, individual animals expressing a fluorescent reporter [33] in the neuron of interest are mounted on a microscope slide and then ablated with a femtosecond laser. The tissue around the neuron is left intact. Finally, to study the activity of individual neurons GCaMP [153,214,3] probes are used. These proteins produce a fluorescent signal dependent on Ca^{2+} concentration in a cell which enables an optical interrogation of polarization states of the neuron.

Using these techniques it was shown that to establish its naive preference for PA14 over OP50 [245], *C. elegans* uses three sensory neurons AWB, AWC and ADF to gather environmental cues that are then processed by several interneurons (AIY, AIZ, AIB and RIM) [31,90] and the output is relayed to a class of motor neurons (SMD) [85,94]. This arrangement allows the animal to translate different gradients of chemical odorants emitted by two different bacterial lawns into a mechanistic choice for the direction of movement. Upon training, the naive circuit is still intact but becomes modulated by the action of an interneuron RIA that makes synaptic connections onto SMD. This modulatory circuit is activated by two antagonistic insulin-like neuropeptides [14] generated in two separate sensory neurons (ASJ, ASI) acting postsynaptically on RIA itself [39].

Furthermore, this mechanism might be more universal than just a choice between OP50 and

PA14 as similar aversive learning behavior was observed when *C. elegans* was given a choice between OP50 and *S. aureus*[156] and *M. nematophilum* and two *P. aeruginosa* strains[239].

2

Phenomenology of collective avoidance

In this chapter we would like to establish that pathogen avoidance is a behavior dependent on community of interacting nematodes. We will approach this problem by showing that the avoidance depends on the worm population density. This was first hinted at ten years ago[64] but no evidence was presented at the time.

We first observed such density dependence in routine survival experiments. In these assays we showed that worms in larger groups were able to outlive animals exposed to the same pathogen in

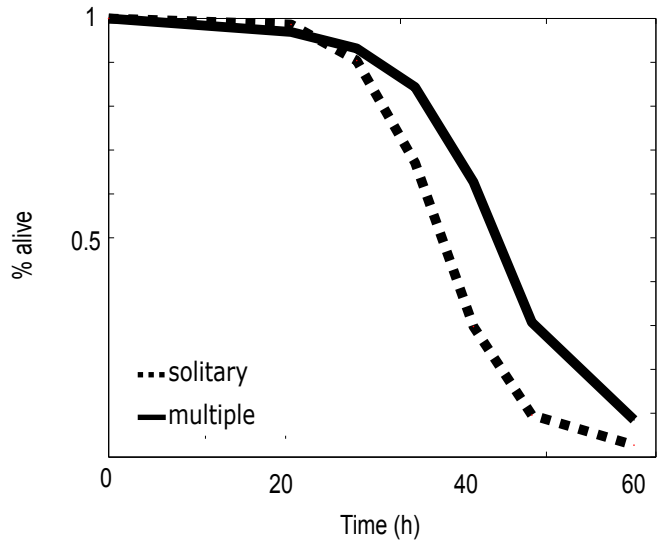

Figure 2.1: Survival curves for solitary and multiple (25-60) N2 worms feeding on PA14 bacteria. The solitary worms die 7h earlier than worms in groups ($p < 10^{-5}$ using a log-rank test).

isolation from other nematodes. More precisely, we carried out a standard slow killing assay[215] on 5 plates carrying 25-60 worms (>160 worms in total) and over 70 plates harboring solitary worms. We tracked the number of surviving animals over time (Fig. 2.1), and found that the half-life of groups of worms was 55 hours and of solitary worms was 48 hours, a 14% difference ($p < 10^{-5}$ using a log-rank test). This suggested that indeed worms exposed to a pathogenic PA14 bacterium in smaller groups were more susceptible to infection.

This density dependence of survival could be explained by two further observations - density dependence of pathogen avoidance, which we will establish in the next section and the relation between pathogen avoidance and survival, which was suggested just a few years ago[195]. In that study *C. elegans* have been exposed to PA14 bacteria in two different conditions: standard lawn (the lawn occupied only a small fraction of the plate, the rest was clear of the pathogen) and the full lawn (the entire surface of the plate was covered with the pathogen) and it was shown that the wild type N2 worms died faster on full lawns. The difference was attributed to the fact that on full lawns *C. el-*

egans were unable to physically avoid the pathogen which increased their exposure and accelerated the infection process. The implication was therefore that pathogen avoidance by itself would increase *C. elegans* survival.

In the following section we will show that increasing the population size of nematodes exposed to the PA14 pathogen accelerates their avoidance through modification of the bacterial lawn.

2.1 Quantitative measurement of pathogen avoidance

To resolve more directly whether different populations of *C. elegans* exhibit different rates of pathogen avoidance we decided to build an automated imaging system. This was necessary because behavioral experiments in living systems are notoriously noisy and we needed to follow many replicates of the same condition simultaneously to counter the statistical noise. Furthermore, pathogen avoidance is an inherently dynamic process where a transition from initial attraction to subsequent repulsion of *C. elegans* to the bacteria happens over a relatively short amount of time and therefore we needed a way to follow the worms at short intervals. A manual assay is sufficient for survival experiments with PA14 where animals are dying over the period of 72h and one is able to count the worms on a few dozen plates every 6-8h. For avoidance experiments we needed to have a much finer time resolution. An added advantage of this approach is that it would allow us to characterize avoidance behavior in an unbiased (independent of personal judgments during manual counting process) and quantitative fashion.

2.1.1 Automatic image analysis system

To this end we constructed an automatic XY stage (propelled in all three directions with stepper motors) with a consumer grade 10MP color camera that traverses an array of 48 assay plates at regular 20-minute intervals (Fig. 2.2). The plates were illuminated by a circular array of red LED lights (as

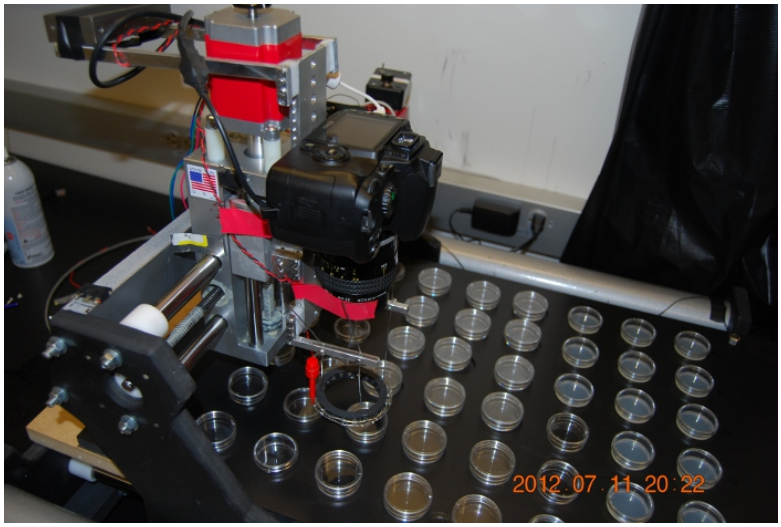

Figure 2.2: XY stage with Nikon D60 camera and LED ring underneath loaded with 48 plates.

higher frequency light negatively affects the worms [231]) permanently suspended under the camera. The entire XY stage was kept in a cardboard box so the LED ring was the only source of light. Before each photograph was taken, the camera-LED rig assembly was lowered so that the LED axes and the focal plane of the camera were overlapping with the surface of the agar. We used a special 55mm close up lens capable of focusing at a distance of less than 25cm which allowed us to optimally utilize the CCD sensor and produce images at 2400x2400 to 2600x2600 pixels in size.

2.1.2 Experimental protocol

We used a modified slow killing (SK) protocol for the media, bacteria and *C. elegans* preparation [215]. The worms were maintained at 25C (with the exception of mutants sensitive to higher temperature which were kept at 15C) on standard NGM plates supplemented with Streptomycin and fed with OP50-1 bacteria. Before an avoidance experiment, *C. elegans* worms were synchronized using a standard bleaching protocol [202] and then allowed to develop for 48h at 25C on NGM plates with OP50-1 bacteria (unless otherwise indicated). In these conditions the wild type N2 worms developed

to adults that were on the verge of starting to lay eggs. The temperature sensitive TJ1060[66] worms (which were used for all experiments in this chapter apart from the survival experiments) were sterile at 25C but otherwise developed at the same rate as N2.

For avoidance experiments with pathogenic bacteria we inoculated a single colony of PA14 in 50ml SK liquid medium (same composition as standard SK medium with the exception of Agar, cholesterol and $CaCl_2$) in a 250ml flask and then incubated overnight (8-16h) on a shaker at 37C. The bacteria were then concentrated 6-10x and 10μl was inoculated into the middle of a standard SK 35mm plate and left to dry for 2h. After that, 1-16 worms were moved using a platinum pick from NGM growth plates to the middle of the PA14 lawn. The recording of the behavior started approximately 30-45min after the worms had been picked.

A standard avoidance experiment produced 110 photographs per plate (5280 images per 48 plates in an experiment) at 20 minute intervals (36h 20min in total). The images were saved in the 12-bit Nikon proprietary format NEF which was subsequently transformed extracted, cropped and saved as a 16-bit TIFF using ImageMagick software. An example of the image at this stage can be seen in Fig. 2.3A.

2.1.3 Image analysis

The remaining analysis is done in Matlab using heuristic semi-automatic workflow. First, the original bacterial lawn is identified (Fig. 2.3B) using the saturation difference between the bacteria and the background. Automatically generated lawns are then verified manually and corrected if necessary. In general the boundary of the lawn is drawn slightly outside ($\sim$50 pixels) the actual lawn so that the worms that are only partially in contact with the bacteria are still counted as being inside the lawn (a typical length of a worm in this set up was 70-100 pixels). Then the workflow identifies areas of the image that changed since previous time point by taking a difference between successive frames and selecting large connected clusters (Fig. 2.3CD). Finally, a list of heuristic subroutines fil-

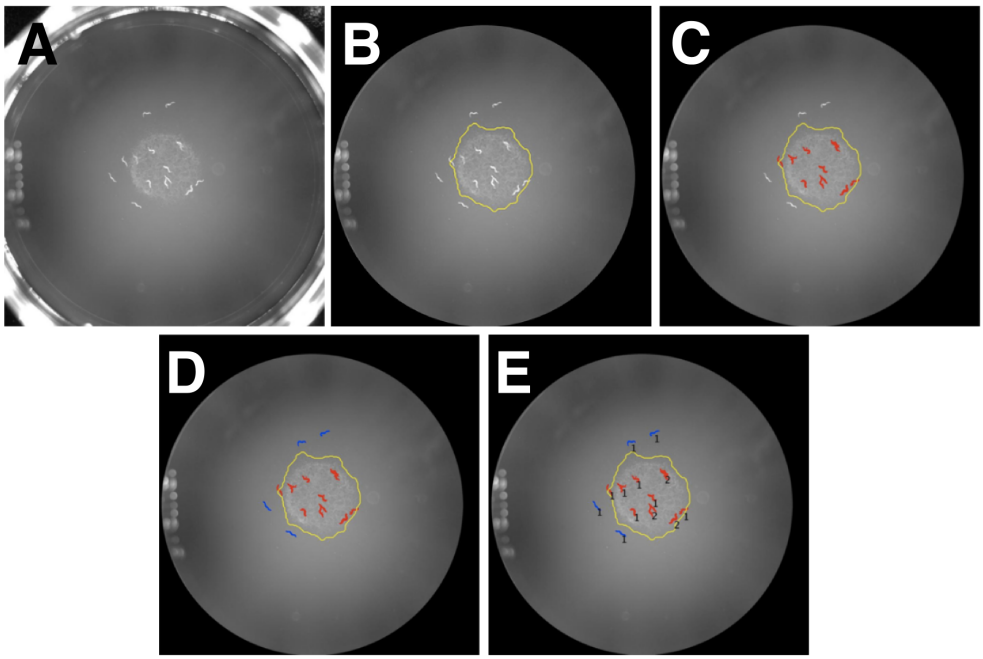

Figure 2.3: Gray scale TIFF images (A) are analyzed using a heuristic Matlab workflow to find the location of the original lawn (B), worms inside (C) and outside (D) the lawn and an estimation of the number of worms in the highlighted areas (E).

ters out areas unlikely to represent worms (based on size, shape and location) and assign a number of expected worms in any given cluster based on area (Fig. 2.3E). Once all worms are counted on all plates at each point of time we define a quantity *avoidance* at a given time point as the fraction of worms on each plate that were found outside the original lawn at that time, averaged over all replica plates. Since worms were initially attracted to the bacteria the kinetics of avoidance behavior was characterized by an increase in avoidance from 0 (Fig. 1.1A) to some final fraction (Fig. 1.1B).

2.1.4 Description of the avoidance assay

Fig. 1.1 shows a characteristic behavior of a worm population on an SK plate seeded with PA14 bacteria. In the first 10-15 hours of the experiment, all (or most) of the worms remained within the confines of the bacterial lawn, and very rarely explored the part of the plate outside. In the initial 2-3 hours worms showed a clear preference for the circumference of the lawn, where it was the thickest (due to a "coffee stain effect"); as the bacterial lawn thickened inside, the worms moved inward. However, in the second half of the experiment an increasing number of worms were found outside the lawn. This "avoidance" behavior is unique to lawns of pathogenic bacteria. On control plates seeded with benign *E. coli* HB101, worms did not leave the lawn for the duration of the experiment (Fig. 2.4). At the same time, noticeable changes occurred in the bacterial lawn, which first became thicker and then, around 15 hours into the experiment, began spreading beyond its original circular shape. As bacteria were unable to swim or swarm in the hard agar medium we attributed their spread to crawling worms (individual bacterial cells could easily attach themselves to the cuticle of the animal) carrying the microorganism outside of the lawn which then multiplied and formed new colonies away from the source.

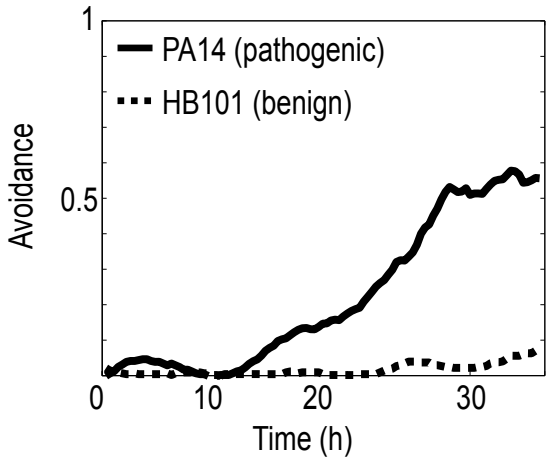

Figure 2.4: Standard avoidance experiment with solitary worms exposed to pathogenic PA14 (solid line) and to benign *E. coli* HB101. Worms avoid the dangerous PA14 but they do not avoid harmless HB101.

2.2 Pathogen avoidance depends on worm population size

To verify if the pathogen avoidance is dependent on the worm population size we placed 1-16 sterile TJ1060 animals on multiple replicate plates and tracked their positions. Fig. 2.5 shows the avoidance curves for each group size. All curves start at 0 (i.e. all worms are within the bacterial lawn) and over 37 hours increase and saturate to a finite fraction f_T at late times (between 24-30 hours, depending on conditions). The value of f_T increases systematically with the increasing number of worms, from 0.57 for isolated worms to 0.90 for a community of 16 worms on a plate.

In all cases when the number of worms per plate was less than 4, all worms remained in the lawn until around $t = 12h$. At this time, worms started to leave the lawn and the avoidance increased to the final level f_T at times larger than $25h$.

The avoidance curves obtained for groups of 4 or more animals on a plate suggest that for such groups lawn leaving occurs in two stages. After $\sim 2h$ small number of animals leave the lawn and the avoidance curve plateaus at an intermediate level whose value and duration depends on the size of the population: from 0.17 for 10h for 4 worms to 0.35 for 7h for 16 worms. Then the avoidance

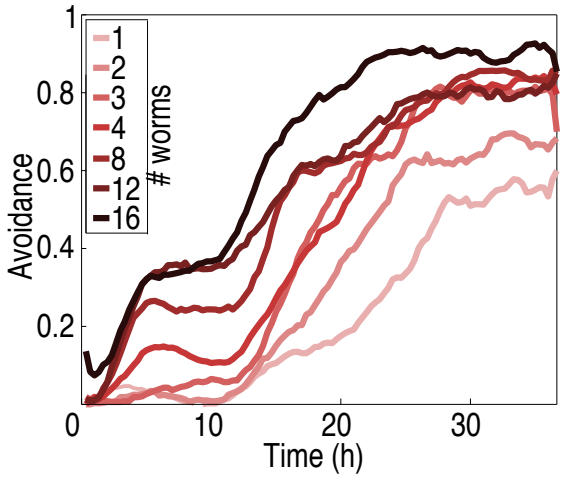

Figure 2.5: PA14 avoidance by worms in groups of 1-16 animals. The pairwise differences between curves are statistically significant, except for the pairs 1/2, 3/4, 8/12 and 12/16.

curves increase to their final saturation values.

To assess whether a pair of avoidance curves is significantly different we used an asymptotic version of the ANOVA F-test[47]. This test allows us to use a Monte Carlo simulation to calculate a probability distribution of the null hypothesis when both curves are Gaussian processes with covariance functions computed from the data. In Fig. 2.5 almost all curves are different pairwise apart from a few adjacent ones (1 and 2, 3 and 4, 8 and 12, 12 and 16).

We conclude that increasing the number of animals on a plate accelerates avoidance - the difference between a single worm and a 16-worm curves at 50% avoidance is 15 hours and it diminishes between successive curves as the population size grows.

2.3 Avoidance signal is deposited in the bacterial lawn

This population size dependency suggests some level of communication between conspecifics. We therefore hypothesized that the avoidance behavior is driven by a pheromone deposited by stressed worms directly into the bacterial lawn. This signal would be detectable by naive worms and it would

accelerate their avoidance. We therefore investigated the avoidance of worms on PA14 lawn which had been pre-exposed to another batch of nematodes.

In the conditioning stage of these experiments (Fig. 2.6A) we placed a ring in the middle of the plate, pipetted an overnight culture of PA14 into the center of the petri dish and after 2h incubation transferred 15-20 worms. The metal ring ensured that the worms stayed in the bacterial lawn for the entire duration of the conditioning phase and also prevented the worms from spreading the bacterial lawn. After 12h, 18h or 30h we carefully removed the ring and all the worms and replaced them with a fresh, naive batch of nematodes (either single worms or groups of 16) and then we tracked the avoidance in time.

Fig. 2.6B (2.6C) shows the avoidance curves for single worms (16 worms). Nematodes begin to avoid the lawn earlier on the conditioned plates and the effect becomes more pronounced as the conditioning time increases. On conditioned plates with 16 worms, half of the population left the lawn up to 12h earlier than in the control. When the worms were isolated on the plates they were able to leave up to 24h earlier on the conditioned plates. This supports the model that naive worms are able to detect the avoidance signal left in the lawn by an independent set of animals.

If a pheromone signal deposited into the lawn drives the avoidance behavior we predicted that it would be more diluted in larger lawns and therefore the animals would start leaving the bacteria later. To further confirm this hypothesis we prepared plates with a standard method and varying amounts of bacteria (4-90μl) that produced lawn sizes: 0.66 to 3.43 times larger than a standard 10μl lawn and placed 8 worms on each plate. Fig. 2.7 shows that indeed avoidance is delayed in larger lawns (the difference between the smallest and largest lawns is 17h) thus further supporting the hypothesis that the avoidance signal resides in the bacterial lawn.

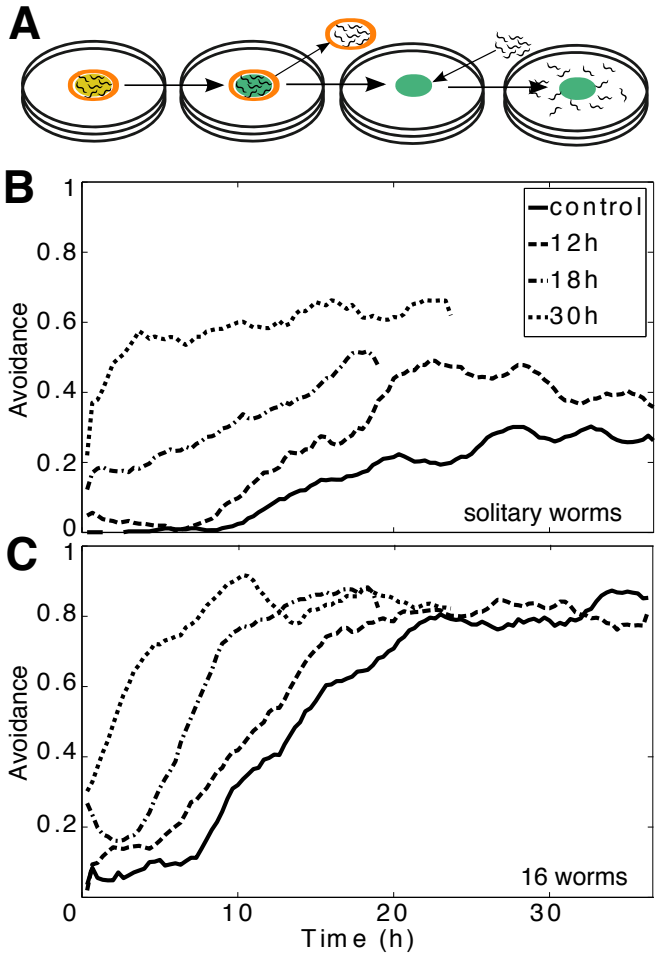

Figure 2.6: (A) Schematic of the preconditioning experiment. (B+C) Avoidance of solitary (B) and groups of 16 (C) worms exposed to lawns preconditioned for 12, 18 or 30 hours. The pairwise differences between curves are statistically significant, except for control/12 hours with 16 worms.

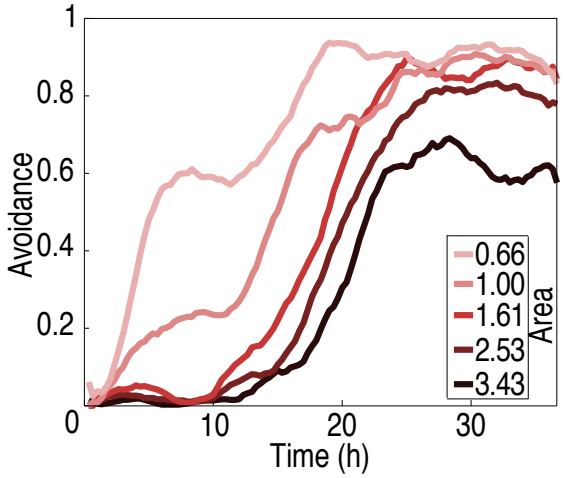

Figure 2.7: Avoidance of 8 worms on lawns of different areas (from 0.66 to 3.43 relative to standard lawns 8-9mm in diameter).

2.4 Worm training effect on avoidance

Instead of pre exposing the bacteria to *C. elegans* we can expose the worms to the pathogen before the assay and then test if there is any memory effect in the avoidance behavior. *C. elegans* have been shown to be capable of aversive learning[245] in a process in which preexposure to PA14 prompts the animals to prefer OP50 bacteria to PA14 despite an innate preference for the latter. A detailed neuronal circuit has been identified that allows the animals to change their preference in a choice assay after 6h-long training on PA14[85] which suggests that the worms are able to learn not to choose the PA14 independently of their conspecifics. Although our avoidance assays never provide a choice of food for the worms this learning mechanism could be relevant in pathogen avoidance as the training on PA14 in choice assays are similar to the first several hours of the avoidance experiments.

We decided to find out whether preexposure of *C. elegans* to PA14 before the assay would accelerate avoidance? Therefore, we investigated worms trained on PA14 for 12h (see Fig. 2.8). We used two different training conditions - in "high density training" approximately 20 worms were trained on standard assay plates with a lawn of radius 8-9mm (at a density of 0.1 worm/mm^2 Fig. 2.8A). In

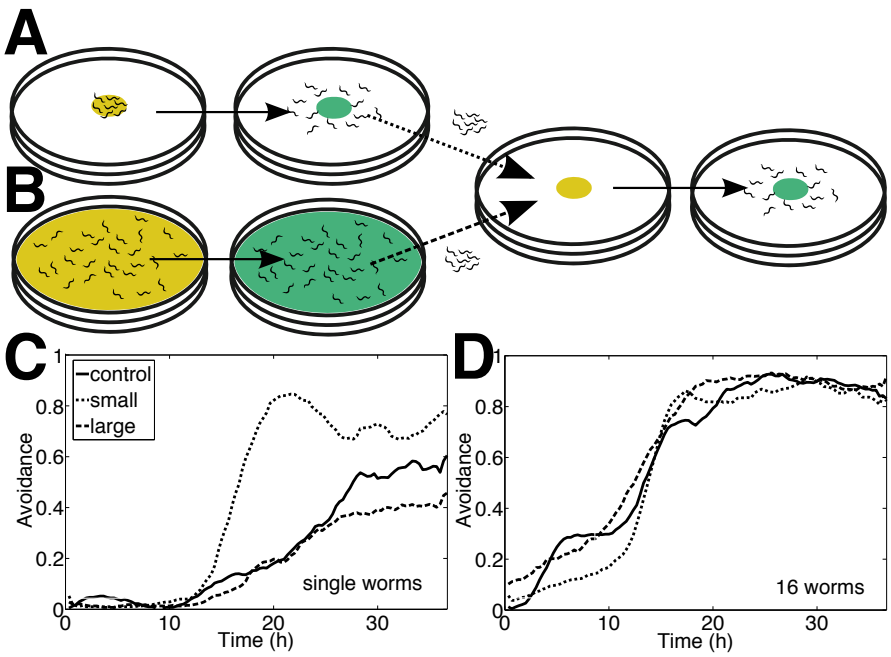

Figure 2.8: Worm training experimental procedure (A). Avoidance of single (B) and 16 worms (C) trained on small (dashed) or large (dashed-dotted) lawns. All (none) of the curves are statistically different in A (B) respectively. Worms pre-exposed to PA14 avoid the bacteria sooner only if the training happened on small lawns and the nematodes are assayed in small groups.

"low density training" 100-200 worms were transferred to 10cm plates fully covered with PA14 (at a density of 0.005 worm/mm^2 Fig. 2.8B). The worm density was 20-30 times smaller on large plates. Once the training was done the worms were transferred to assay plates (single worms or in groups of 16) and their behavior recorded.

The worms trained in high density conditions and then separated and placed individually on assay plates were the only ones that showed any effect of training - they were leaving the lawn 10h before the naive control (Fig. 2.8C). Groups of 16 worms trained in any condition (Fig. 2.8D) did not differ from control which suggests that the benefits of preexposure may be negligible compared to a cooperation of the group in covering the bacterial lawn with the pheromone. The effects of training were also absent in single worms trained on large lawns (Fig. 2.8C). This suggests that for an effective preexposure the training conditions must include a larger density of worms and the ability to leave the lawn.

2.5 Bacterial avoidance is a dynamic process

Worms integrate several environmental cues that determine their choice of whether to remain in the original lawn or leave (e.g. presence of bacterial food and any potential pheromonal cues). Additionally, the associative learning experiments [245] show that exposure to PA14 can change their preference over longer periods of time. A question arises how permanent are the choices made by the animals: once a nematode leaves the bacterial lawn does it ever come back? If so, how long does it take it to revert its preference. We can answer these questions by tracking a single worm per plate which will allow us to record its specific history.

The data for each worm is a binary time series indicating whether the worm was found inside or outside the lawn. We divided the entire population of isolated worms into animals that were within the lawn ("in population" p_T^{in} - green curve in Fig. 2.9A) and outside of it ("out population"

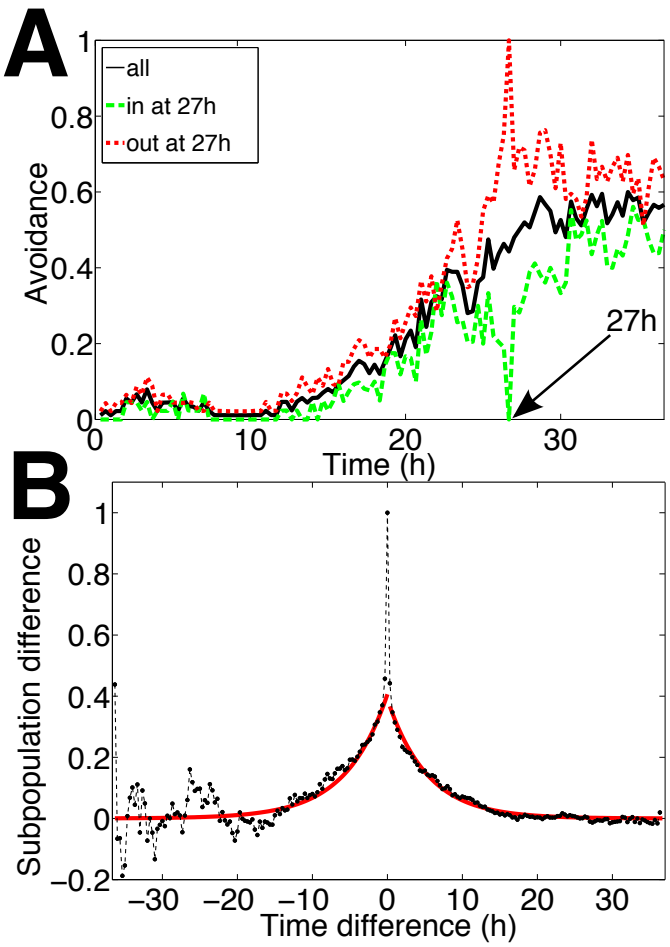

Figure 2.9: (A) Avoidance curves for subpopulation of isolated worms that were inside (dashed, green) or outside (dotted, red) at time 27h post infection (division time T=27h). (B) Averaged difference between the "in" and "out" curves for all division times 0h$<$T$<$37h plotted as a function of time difference from T. The red curves are exponentials decaying with $5.5h$ time constant. *C. elegans* enter and leave the lawn continuously and the probability that a given worm switches between in and out state decays with two distinct timescales: a short $<$20 min and long $\sim$5.5h.

p_T^{out} - red curve in Fig. 2.9A) at a given time point (e.g. $T = 27h$ in Fig. 2.9A) . Then we followed these two subpopulations separately in time and plotted their respective avoidance curves $p_T^{in}(t)$ and $p_T^{out}(t)$. The difference of these two curves $P_T(t) = p_T^{out}(t) - p_T^{in}(t)$ was equal to unity at $t = T$ by construction and then decayed to zero at $t = 0h$ and $37h$.

We calculated P_T curves for all $0h < T < 37h$ and plotted them with $t = T$ as the origin (i.e. each plot was equal to one at the origin). Fig. 2.9B shows the average of curves constructed in this way - the mean curve peaks at unity at the origin and then decays to zero on both sides. There is significantly more noise at negative end of the plot because at the early times of the experiment almost all worms are inside of the bacterial lawn. The "out population" is very small which results in higher variability. The decay from the peak happens in two stages. First, within the 20 minutes the curve decreases by 55%, then it decays with a single exponential on both sides with a time constant of approximately $5.5 \pm 0.5h$.

When we performed the experiment with 16 worms on each plate (after 12h we removed all worms that were outside of the lawn at that instant and then followed the behavior of the rest) the system equilibrated within the first 20 min that it took between removing the animals and loading the plates into the XY stage.

This analysis shows that there are two distinct time scales at which *C. elegans* change their location between in and out of the lawn. At short time scale (<20min) over half the animals will change their location with respect to the lawn. However, the remaining nematodes will be more likely to stay and their preference will decay exponentially with the time constant of 5.5h. When there are multiple worms on the plate the system equilibrates completely within 20 min.

The fact that the worms move in and out of the bacterial lawn explains how the bacteria are able to spread across the plate. In hard agar conditions the PA14 bacterial are unable to move (by swimming or swarming): the original lawn does not expand when there are no worms present. When the nematodes move through the lawn, individual bacterial cells are able to attach themselves to the cu-

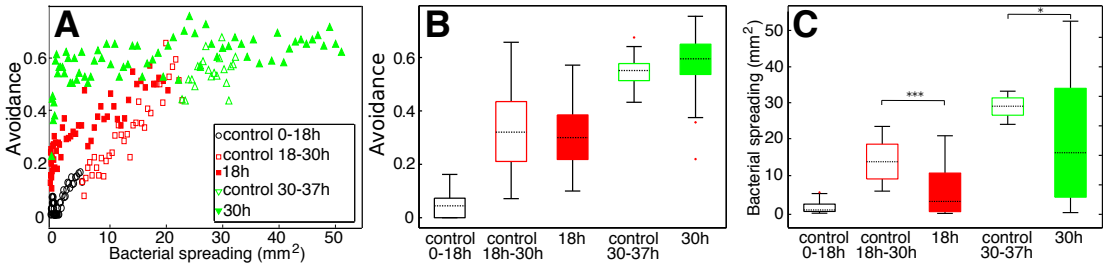

Figure 2.10: Correlation between avoidance and bacterial spreading for a standard avoidance assay (open markers) as well as for 18h and 30h conditioning assays (red and green solid markers respectively) for solitary worms. (B-C) A box plot showing distributions of avoidance (B) and bacterial spreading (C) for avoidance and conditioning experiments in (A). Single and three stars indicate statistically significant difference at p<0.02 and p<10-6 level according to ANOVA. The lawn occupancy fraction depends on how long the lawn was exposed to the *C. elegans* and is independent of how much the lawn was spread.

ticle of the worms and hitch a ride to areas of the plate free of bacterial occupation. There they can seed a new colony that will grow over the course of several hours to be visible in our experimental setup. Once this external lawn appears *C. elegans* might be attracted away from the original lawn to the new sources of food. This rises the question how much does the growing lawn contribute to the measured avoidance behavior. The next section (and the following chapter) will show that avoidance precedes the lawn expansion and in fact is a driving force behind it.

2.6 Bacterial spreading is a consequence of avoidance

Finally, we can answer a question about the relation of pathogen avoidance and bacterial spreading. In principle the worms affect the bacteria on the plate in two ways. First, are the chemical or morphological modifications to the lawn which we have discussed in previous sections. The second is the bacterial spreading caused when microorganisms adhering to the cuticle seed new colonies around the plate when worms leave the lawn.

This leads to a question - do the worms avoid the lawn because of the deposited pheromone or simply because they are attracted to new sources of food after the bacteria were spread.

To answer this question we compared three experiments. A standard avoidance assay as described in the first chapter of Results (Fig. 1.1) and 18h and 30h conditioning experiments (Fig. 2.6). We present the data in Fig. 2.10A: hollow markers correspond to the control experiment, red full squares to 18h conditioning and green full triangles to 30h conditioning. Each marker corresponds to one time point in the experiment for single worms. The vertical axis is the avoidance as shown in Fig. 1.1 and Fig. 2.6B. The horizontal axis measures "bacterial spreading" which is an average between all replicate plates of a total lawn area minus the original lawn area at a given time.

In the standard experiment there is no limitation to worms' movement and both effects (modification of the original lawn and spreading) can take place. Over time the lawn size increases and avoidance is linearly correlated with the amount of spreading (hollow markers in Fig. 2.10A). On the other hand, both conditioning experiments, prevent the worms from leaving the original lawn for 18h or 30h before the start of the assay through the use of a metal ring. In that time, the conditioning worms are only able to affect the original lawn. Therefore, if spreading is driving the avoidance dynamics we should see no difference between the control and conditioning experiments because in both cases the lawn spreading is small at the start of the assay. However, if avoidance is affected only by the state of the original lawn then we should see increased avoidance in the conditioning experiments at even small values of spreading as the worms stay clear of the original lawn but the bacteria did not have time to grow outside of it.

Fig. 2.10A shows that 30% (50%) of the worms left the original lawn in 18h (30h) conditioning experiment when the new lawn had spread to less than 5mm^2. At the same level of spreading only 10% of animals had avoided the original lawn in the control experiment.

Furthermore, the extent to which the worms avoid the lawn in 18h (30h) conditioning experiments matches very closely the avoidance in control between 18h-30h (30h-37h). The distributions of avoidance in these experiments are shown in Fig. 2.10B. Using one way ANOVA test, the p-value between 18h conditioning and 18h-30h control distribution of avoidance is 0.58 and between 30h

conditioning and 30h-37h control - 0.076 (all other p-values are below 0.05). Therefore we cannot reject the null hypothesis that these avoidance distributions are the same. In turn, the corresponding p-values for the distributions of areas of spreaded lawns (Fig. 2.10C) are $1.6 \cdot 10^{-8}$ (18h conditioning and 18h-30h control) and 0.018 (30h conditioning and 30h-37h control).

These different lawn area distributions between control and conditioning experiments lead to similar avoidance and therefore these two effects are likely decoupled. We conclude that the driving force behind avoidance are chemical or morphological changes to the original lawn and the bacterial spreading is merely a consequence of avoidance itself.

2.7 Conclusions

In this chapter we developed a new automatic imaging system that measures the distribution of *C. elegans* nematodes in a slow killing assay. Using this setup we showed that the rate at which the worms evacuate a lawn of pathogenic bacteria depends on the density of animals. This novel phenomenon suggests some form of communication between conspecifics. We demonstrated that it is indirect and it occurs through the bacterial lawn, as the alarm signal left by one batch of worms can be detected by an independent naive set of animals.

Training of the animals (pre exposing them to PA14 before the assay) has no clear effect on the collective avoidance suggesting that aversive learning behavior[245] is an independent phenomenon. Furthermore, we showed that the avoidance is a dynamic process with worms entering and leaving the bacterial lawn multiple times throughout the experiment with two distinct timescales. This suggests that they may be balancing a perceived danger (quantified by the level of the alarm pheromone) and hunger to adjust the time they spend outside and inside the lawn. We also showed that bacterial spreading beyond the original lawn (which becomes more prominent at later stages of the experiment) is a byproduct of the nematodes' movement.

These results support the hypothesis that the worms are capable of depositing a warning signal as a consequence of infection. The nature of this signal is unclear but it could either be a chemical signal produced by the worms and directly detected by other nematodes or the *C. elegans* can have an effect on the bacteria themselves (through better aeration of the lawn or PA14 could detect the chemicals produced by the worms) which prompt the microorganisms to repulse the worms from each other indirectly.

2.8 Methods

2.8.1 Strains and media

We used TJ1060 (*spe-9(hc88)I; fer-15(b26)II*) temperature sensitive, sterile strain in this chapter. Worms were maintained at 15C on high growth media (HGM) plates[101] supplemented with 180 μg/ml streptomycin (to avoid PA14 contamination), and fed with streptomycin-resistant *E. coli* OP50-1 using standard protocol[101]. Bacterial strains used were *Pseudomonas aeruginosa* PA14 (a gift from F. Ausubel) and *Escherichia coli* HB101 (a gift from S. Mango). *E.coli* were grown overnight at 37C in LB medium and *P. aeruginosa* in a liquid SK medium (0.35% peptone, 0.3% NaCl, 1 mM $MgSO_4$, 25 mM KH_2PO_4).

2.8.2 Worm preparation

All worm strains were synchronized by hypochlorite treatment and first larval stage (L1) arrest for 12-16h[101]. Arrested L1s were transferred onto standard 6cm NGM plates (supplemented with 180 μg/ml streptomycin) seeded with OP50-1. Worms were allowed to develop for 48h at 25C. In these conditions $>$ 95% reached adulthood and were sterile.

2.8.3 Avoidance assays

P. aeruginosa and *E. coli* avoidance assays were performed on solid SK medium [215]. We used 3.5cm plates with 4ml of appropriate media. To prepare PA14 plates, 10μl (or 4μl, 10μl, 30μl, 60μl and 90μl in case of variable lawn area version of the experiment) of 6x concentrated overnight culture were spotted in the middle of an SK plate. Plates were left to dry in a laminar flow hood for 1h with open lids. The lids were then replaced and the plates were incubated at room temperature for an additional hour. For control experiments, 10μl of E. coli HB101 culture were spotted in the middle of an SK plate, and incubated overnight at 37C. Plates were then allowed to cool down for 30 minutes at room temperature. Each avoidance curve represents an average avoidance on 42, 27, 18, 24, 12, 6 and 6 plates for solitary, two, three, four, eight, twelve and sixteen worms respectively. At least two independent repeats were performed. Each avoidance curve is smoothed with a moving window of 9 frames (3h).

2.8.4 Survival assay

PA14 slow killing assay was performed according to standard procedure [215]. Briefly, a 10μl of an overnight culture of PA14 in LB was spotted onto a center of an SK plate. Plates were then incubated at 37C for 24h and then an additional 24h at 25C. 4 plates with 28-54 worms each (167 worms in total) were used and 73 plates with solitary worms. Dead and alive worms were counted every 8 hours for 3 days.

2.8.5 Lawn conditioning assay

A stainless steel washer (obtained from McMaster-Carr, catalog number: 92141A030; washed and autoclaved before every use) was placed in the middle of an SK plate. 10μl of 6x concentrated overnight culture of PA14 were carefully dropped into the middle of the ring (taking care that the liquid does

not touch it). After an hour of drying in laminar flow hood (without the lids) and an hour-long incubation (with lids on) at room temperature, 15-20 worms were placed onto bacteria and plates were incubated at 25C. At the end of the conditioning period (12, 18 or 30 hours), the rings were carefully removed and all worms were picked. If the procedure damaged the agar in any way, or if any worm was found outside of the inner diameter of the ring, the plate was discarded. Naive worms were subsequently transferred onto the conditioned lawn, and their avoidance was monitored. A control conditioning experiment follows the same procedure as PA14 avoidance experiments, except that the plates were incubated for the duration of the conditioning period with no worms.

2.8.6 Image acquisition and analysis workflow

Image acquisition hardware was based on a CNC Router Fireball V90 (www.probotix.com) with 3-side step motor driver kit, anti-backlash and RF Isolated PBX-RF Breakout Board. The CNC router gantry carried a Nikon D60 (or D40x interchangeably) consumer-grade camera with a Nikon 55mm F3.5 Micro- Nikkor close up lens. Both the XY stage and the camera were controlled by a computer using Linux- CNC software (www.linuxcnc.org) and gPhoto2 command-line interface (www.gphoto.org). The entire system, capable of imaging up to 48 plates, was enclosed in a dark box in a temperature-controlled room (24C ± 1). Illumination was provided by 20 red LEDs wired in parallel and mounted on a plastic ring 60mm in diameter. The diodes were suspended under the camera at the level of the agar surface. Plates were thus in the dark during most of the experiment, and were only illuminated briefly when imaged. Suitably cropped images were saved at 2600x2600 or 2500x2500 resolution and 12-bit depth. A semi-automatic heuristic image analysis workflow, written in Matlab (Mathworks, MA), identified the location of the original bacterial lawn, positions of individual worms and areas where bacteria were spread.

2.8.7 Statistical analysis

Statistical test for similarity between the avoidance curves has been performed using an asymptotic version of the ANOVA F-test[47]. We used 10^5 Monte Carlo simulations to obtain empirically the asymptotic distribution under a null hypothesis that the two curves are taken from the same Gaussian process with statistics taken from the data. The results reported indicate a threshold on statistical significance at p-value<0.05.

3

Phenomenological model

In this chapter*, we investigate more closely the dependence of pathogen avoidance on the growing external lawn and show that indeed the worms are repulsed from the original lawn and attracted to the new bacteria. This effect could be attributed to either a repulsive pheromone deposited by stressed worms that drives them away from the old lawn or putative attractive nature of freshly grown lawns. To resolve which effect is behind the pathogen avoidance we focus on the first 10

*This chapter is the result of work done in collaboration with Iulia Neagu

hours of the avoidance assays where no spreading has occurred yet (the newly seeded bacteria did not have time to grow) but the worms already begin to abandon the original lawn in a density dependent manner. We present a 4-state phenomenological model that we use to reproduce the qualitative features of the avoidance assays and show that a pheromone deposited into the lawn by the worms can indeed explain the initial evacuation of the lawn.

3.1 Bacterial spreading is correlated with avoidance

As we have discussed in Chapter 2, there are two processes taking place during an avoidance experiment: a chemical (or morphological) modification of the original lawn and bacterial spreading. We have shown using the conditioning experiments that the driving force behind the pathogen avoidance is the effect that the worms exert on the original lawn and the bacterial spreading is merely a consequence (Fig. 2.10) of their avoidance. However, at later times ($t > 15h$) the growing bacteria outside of the lawn present an abundant alternative source of food that will attract the nematodes. Therefore, this external lawn warrants a more detailed analysis.

Fig. 3.1A shows the ratio of areas of the growing external lawn to the original lawn for different numbers of worms N. The dashed lines represent a numerical fit of these experimental curves to:

$$x(t) = \frac{\text{external lawn}}{\text{original lawn}} = \frac{\mathcal{A}_{max} t^3}{t_{1/2}^3 + t^3}, \tag{3.1}$$

where $\mathcal{A}_{max}$ is the asymptotic value of the function and $t_{1/2}$ is the time when it reaches $\mathcal{A}_{max}/2$. The values of both parameters for each curve are shown in Fig. 3.1BC and they can be fitted to linear and logarithmic functions respectively.

One of the drives behind the nematodes' behavior is hunger which compels the animals to stay on food. In our experiments the worms are found on either the original lawn or the newly grown bacteria most of the time. Therefore, quantifying the size of the new lawn allows us to to test whether

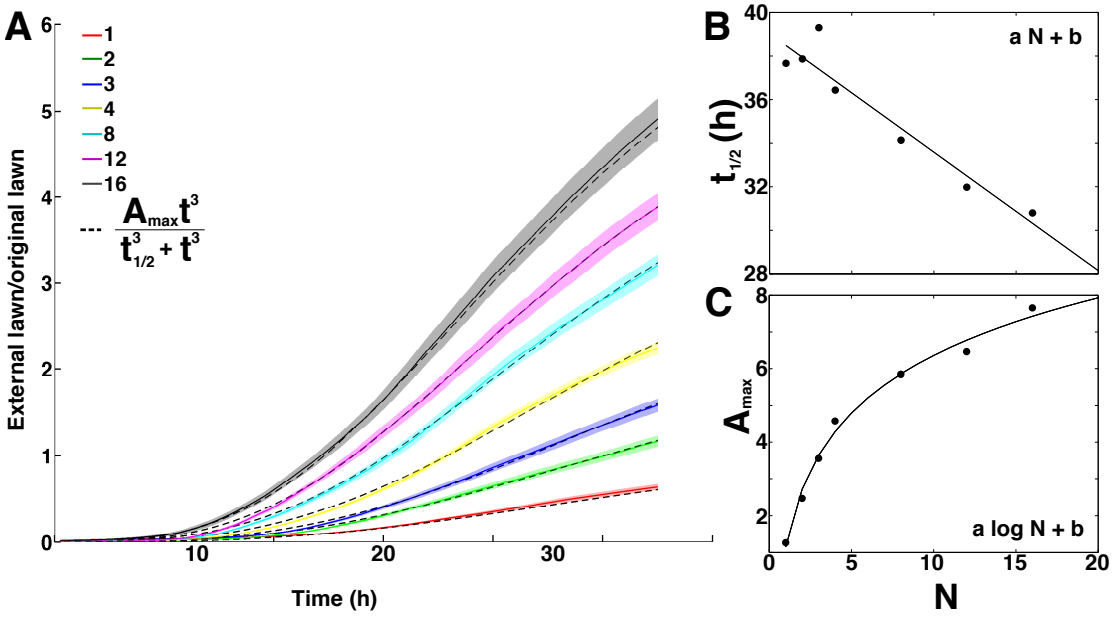

Figure 3.1: (A) Average and the standard error of the external lawn to original lawn ratio for various worm number N avoidance experiments. The dashed curves show a fit to $\mathcal{A}_{max}t^3/(t_{1/2}^3+t^3)$ curve where the parameters are fitted to a linear (B) and a logarithmic (C) equations.

the worms are attracted to the original and new lawns equally. To address this null hypothesis we plotted all data points from the avoidance experiments for various worm numbers in Fig. 3.2. The horizontal axis is the ratio x of external to original lawn sizes (same as in Fig. 3.1A), the vertical axis is the fraction of worms *outside* the original lawn. If the worms see no difference between old and new bacteria then this fraction should be simply proportional to the fraction of the original lawn to the total bacterial lawn at the time: $1/(1+x)$.

The red curve in Fig. 3.2 shows a $a/(a+x)$ best fit for the avoidance data. Rather than a unity, the parameter $a = 0.5074$ (at $r^2 = 0.95$) indicating that the worms are two times less likely to be found on the original lawn than on the new one (per a unit of area). Which supports the hypothesis that the original lawn is less attractive to the worms either because the repulsive effects of the putative alarm pheromone or because worms prefer to feed on the freshly grown lawn.

We can use the fitted relation between avoidance and external lawn size to reproduce the original

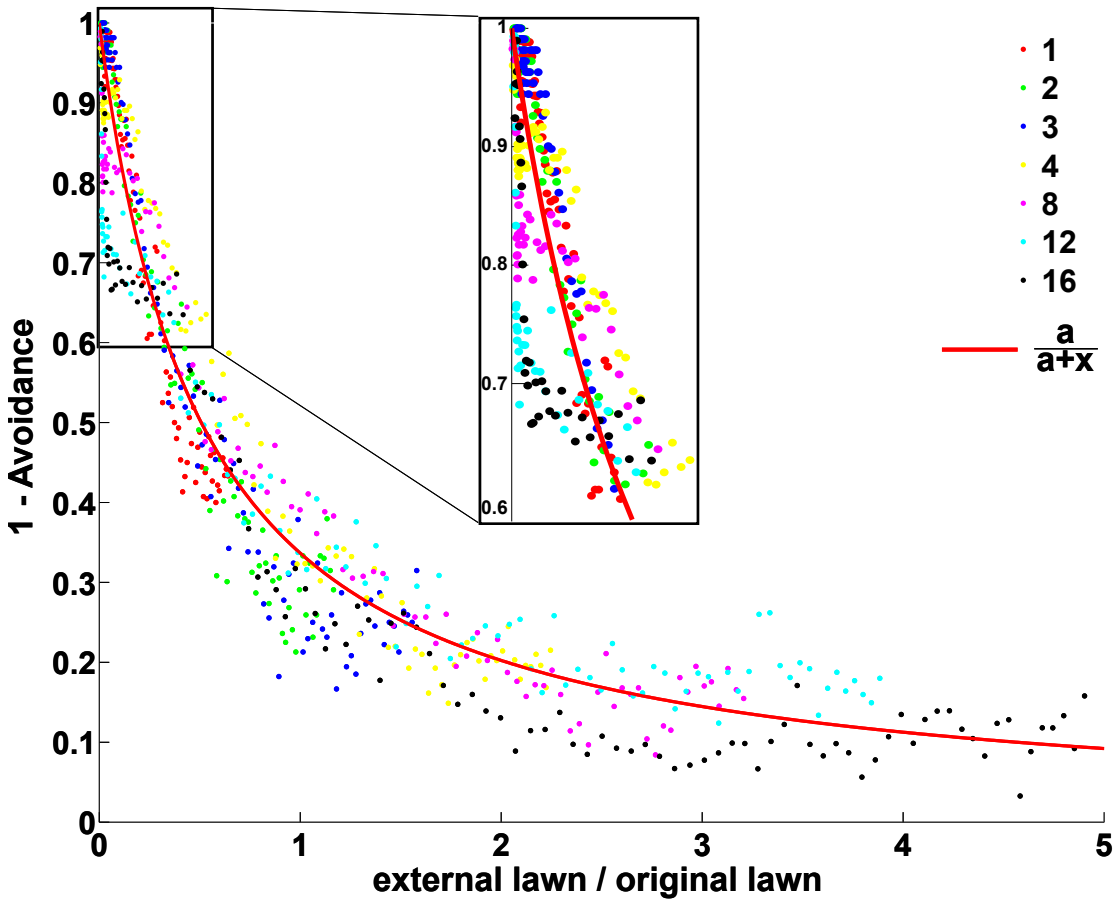

Figure 3.2: Each point corresponds to a single time point of a given avoidance experiment with avoidance and the size of the external lawn measured at that moment. The red curve is a best fit $a/(a+x)$ with $a = 0.5074$. x is the ratio between external and original lawn sizes.

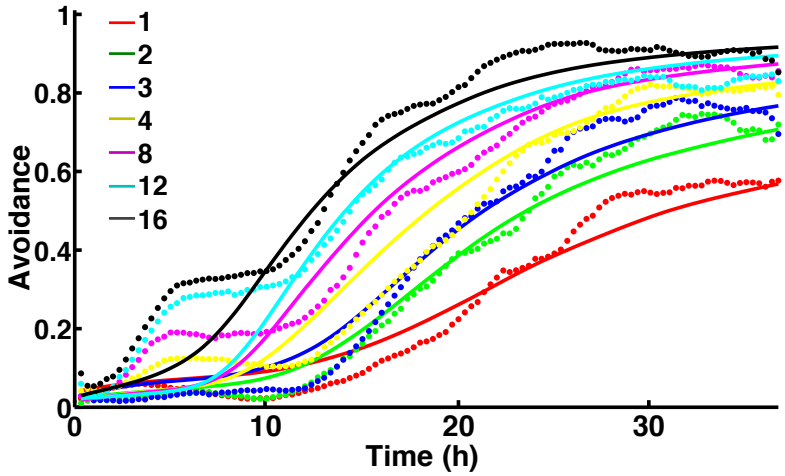

Figure 3.3: Avoidance calculated based on the best fit model to the external lawn size from Fig. 3.2 (continuous curves) compared to the data (dotted curves).

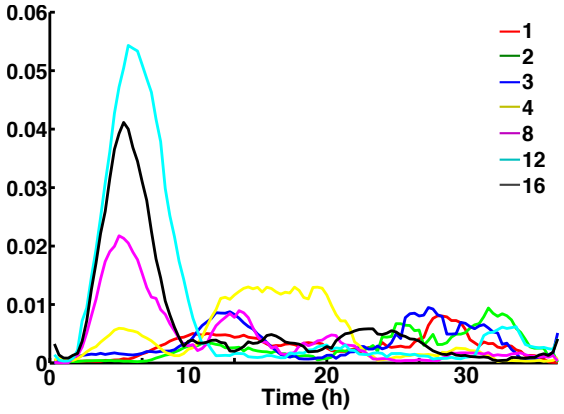

Figure 3.4: The square of the difference between the continuous (empirical model) and dotted (measurement) curves from Fig. 3.3. The maxima for $N > 4$ occur at around 5.5h.

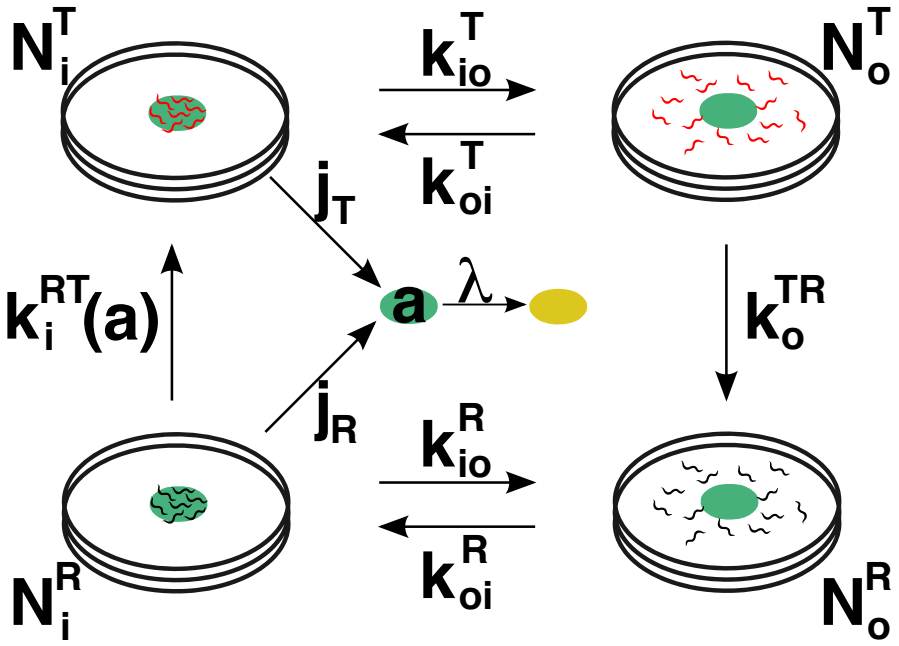

Figure 3.5: A 4-state model of initial pathogen avoidance. Worms could be relaxed (black, R) and upon exposure to the alarm pheromone tense (red, T) and inside or outside the lawn.

avoidance curves. Fig. 3.3 shows the prediction based on the measured area of the lawn (continuous curves) plotted agains the data (dotted curves). The curves correctly reproduce the overall dynamics throughout the experiment with the exception of initial 10 hours for higher N. This discrepancy is more obvious in Fig. 3.4 which shows the square of the difference between the model and the data. In this initial stage of the experiment the worms are already evacuating the lawn but the bacteria that they carried outside with them have not had the time to grow yet. Therefore, we need another approach to model the first phase of the dynamics. This model will also help us establish causality between the external lawn size and avoidance.

3.2 Initial avoidance

Fig. 3.5 shows a 4-state model with possible transitions. The worms could be either inside (*i*) or outside (*o*) the lawn and their interaction with the alarm pheromone could change their status from *relaxed* (*R*) to *tense* (*T*). The worms in either status could move from the lawn to the outside but tense worms could only recover back to the relaxed state outside the lawn (k_o^{TR}) and the relaxed ones could become tense inside the lawn (k_o^{RT}). This last transition is dependent on the concentration of the pheromone in the original lawn and we have chosen a Hill function (Eq. 3.2) to represent it, because we expect that the interaction between the ascaroside (a ligand) and a a putative GPCR receptor will follow Michaelis-Menten kinetics. The worms in T (R) status produce the pheromone (only within the lawn) at a rate j_T (j_R) and it can decay at a rate λ (Eq. 3.3).

$$k_i^{RT}(a) = \frac{k_i^{RT}\,(a/a_\circ)^n}{1 + (a/a_\circ)^n} \tag{3.2}$$

$$\dot{a} = j_T N_i^T + j_R N_i^R - \lambda a \tag{3.3}$$

The detailed rate equations are:

$$\dot{N_i^T} = k_i^{RT}(a) N_i^R + k_{oi}^T N_o^T - k_{io}^T N_i^T \tag{3.4}$$

$$\dot{N_i^R} = k_{oi}^R N_o^R - k_{io}^R N_i^R - k_i^{RT}(a) N_i^R \tag{3.5}$$

$$\dot{N_o^T} = k_{io}^T N_i^T - k_{oi}^T N_o^T - k_o^{TR} N_o^T \tag{3.6}$$

$$\dot{N_o^R} = k_o^{TR} N_o^T + k_{io}^R N_i^R - k_{oi}^R N_o^R \tag{3.7}$$

Using the least squares fit for the first 13 hours of avoidance curves we found the values of the parameters. The model predictions are shown in Fig. 3.6. In these solution the worms start out in the relaxed state inside the lawn. Within a few hours a steady state develops where only a fraction of the

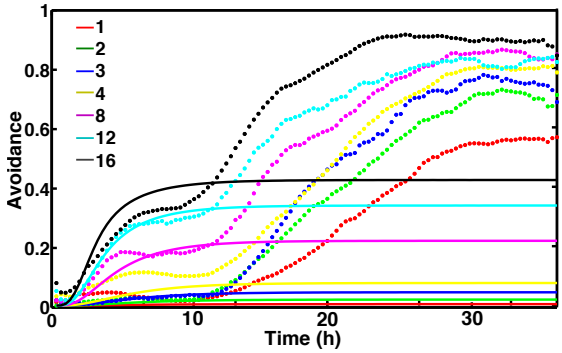

Figure 3.6: Avoidance calculated based on the phenomenological model from Fig. 3.5 (continuous curves) compared to the data (dotted curves).

original population is required to sustain a constant, non-zero level of the pheromone inside the lawn which in turn keeps away some nematodes. Interestingly, this model was able to capture the initial dynamics of the avoidance experiments much better than the earlier approach based on the bacterial spreading. This is more visible in Fig. 3.7 which is showing the square of the difference between the curves in Fig. 3.6 (continuous curves) and for comparison reproduces the results of the previous model from Fig. 3.4 (dotted curves). Additionally, this particular parameter fit to the data predicts that $\frac{j_T}{j_R} \approx 2.1$, i.e. the rate of alarm pheromone production of tense ('alarmed') worms is twice as that of relaxed ones and

$$\frac{k_{oi}^R}{k_{io}^R} \approx 29.3 \gg \frac{k_{oi}^T}{k_{io}^T} \approx 2.6,$$

i.e. relaxed worms are ten times more inclined to enter the lawn than the tense ones.

To see how sensitive are these model predictions to individual parameter values in that particular fit we can focus on the first 10 hours of the experiment and integrate the area under the avoidance curve in that time window. The points with error bars in Fig. 3.8 show the measured experimental areas under the curves. The black line shows the model predictions with fitted parameters. The green continuous (dashed) curves show model predictions for changing values of j_R from 2 to 10 times (1/2 to 1/10 times) the fitted value. The red line show the same curves for changing

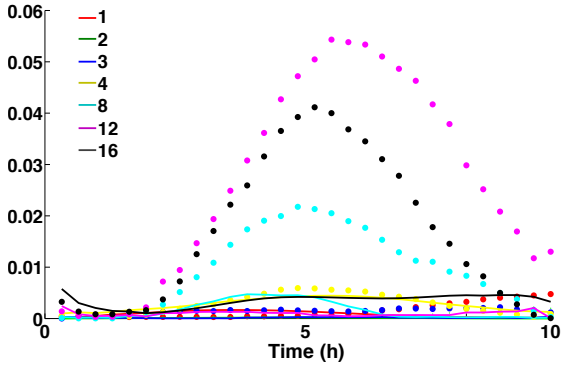

Figure 3.7: The square of the difference between the continuous (phenomenological model) and dotted (measurement) curves from Fig. 3.6 for the first 10 hours of the experiment. The dotted curves are for comparison to the empirical model based on the external lawn size from Fig. 3.4.

j_T. The clustering of the red dashed curves in Fig. 3.8 suggests that the rate of production of the alarm pheromone by tense worms (i.e. those that are affected by the signal) is insignificant, as long as it is very low. This means that those worms do not contribute significantly to the level of the pheromone. This may be because the tense worms are driven away from the lawn and effectively do not spend enough time there to affect the concentration of the signal. However, if the pheromone secretion for either worms (j_T or j_R) is upregulated, the level of avoidance increases quickly. For relaxed worms this holds true for all N, for tense worms it only becomes significant for $N > 4$. Overall, the model is very sensitive to j_R as they are the only ones that keep up the elevated levels of the pheromone and j_T could effectively be set to zero.

Fig. 3.9 shows the dependence of the model on the rate of leaving (k_{io}^T) and entering (k_{oi}^T) the lawn by tense worms. These parameters have a very limited effect on avoidance as decreasing the entering rate and increasing the leaving rate will not change the model predictions. Therefore, we could set $\frac{k_{oi}^T}{k_{io}^T} \approx 0$ which would mean that once the worms become alarmed by the pheromone (tense) they will leave at a constant rate and remain outside until they transition into the relaxed state. Similarly if we analyze the entering (k_{oi}^R) and leaving (k_{io}^R) rates for the relaxed worms (Fig. 3.10) we see that the model is sensitive to k_{oi}^R but not to k_{io}^R (as long as they are low enough). Therefore k_{io}^R, k_{oi}^T and

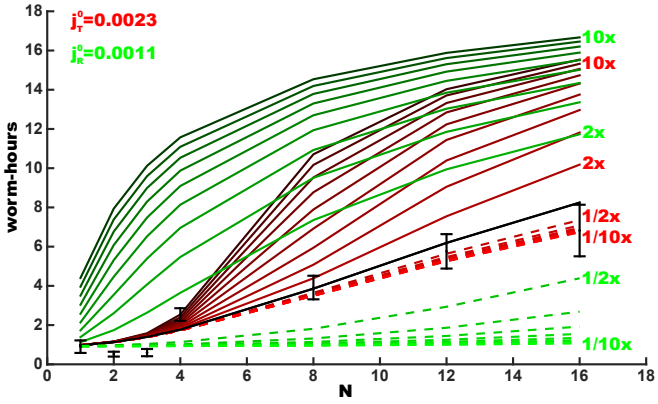

Figure 3.8: Cumulative avoidance in the initial 10 hours as a function of j_T and j_R. The points with error bars show the measured experimental areas under the curves (with standard error of the mean). The black line shows the model predictions with fitted parameters. The green continuous (dashed) curves show model predictions for changing values of j_R from 2 to 10 times (1/2 to 1/10 times) the fitted value. The red line show the same curves for changing j_T.

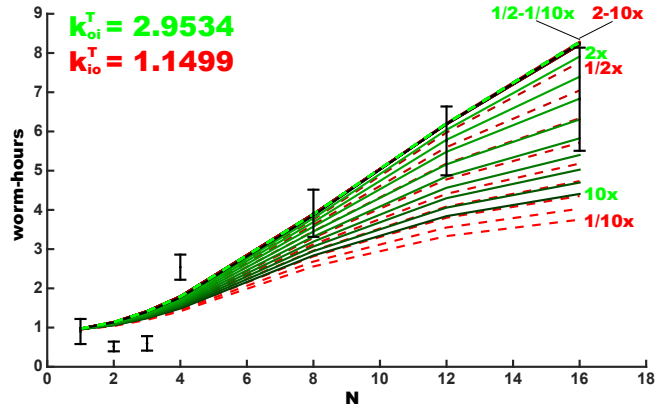

Figure 3.9: Cumulative avoidance in the initial 10 hours as a function of k_{oi}^T and k_{io}^T. The points with error bars show the measured experimental areas under the curves (with standard error of the mean). The black line shows the model predictions with fitted parameters. The green continuous (dashed) curves show model predictions for changing values of k_{oi}^T from 2 to 10 times (1/2 to 1/10 times) the fitted value. The red line show the same curves for changing k_{io}^T.

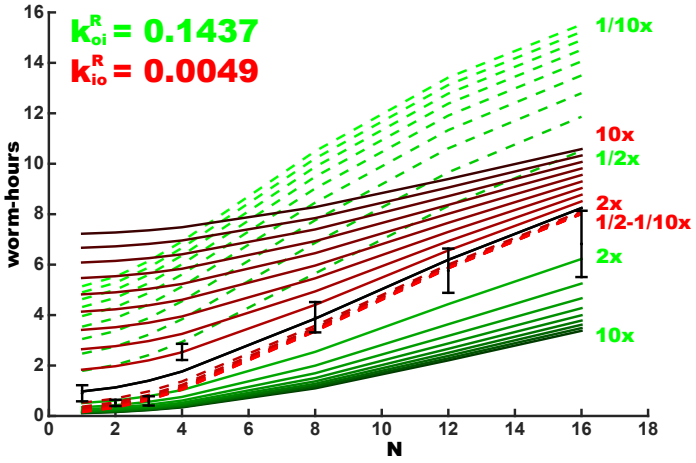

Figure 3.10: Cumulative avoidance in the initial 10 hours as a function of k^R_{oi} and k^R_{io}. The points with error bars show the measured experimental areas under the curves (with standard error of the mean). The black line shows the model predictions with fitted parameters. The green continuous (dashed) curves show model predictions for changing values of k^R_{oi} from 2 to 10 times (1/2 to 1/10 times) the fitted value. The red line show the same curves for changing k^R_{io}.

j_T do not significantly affect the initial amount of cumulative avoidance. Thich would suggest that we could set them all to zero and be able to reduce the model to a 3-state system (relaxed worms inside the lawn, relaxed and stressed worms outside), however closer inspection of the avoidance curves in that case reveals that the fit of the three state model is significantly worse qualitatively. Hence, these three parameters play more subtle roles in this model, beyond what could be captured by cumulative avoidance as analyzed in Figures 3.8-3.10.

Despite these shortcomings, we can use the 4-state phenomenological model to reproduce some features of the conditioning experiments. In the first stage of those assays the worms are prevented from leaving the lawn, therefore we set $k^R_{io} = k^T_{io} = 0$ which resulted in the substantial buildup of the pheromone in the lawn compared to the standard avoidance assays (Fig. 3.11).

Then using that final elevated level of the alarm signal as the initial condition we generated avoidance curves for the conditioning assay. In Fig. 3.12 continuous lines show the model prediction overlapping the data points from the 30h conditioning experiments in Fig. 2.6 (red - solitary worms,

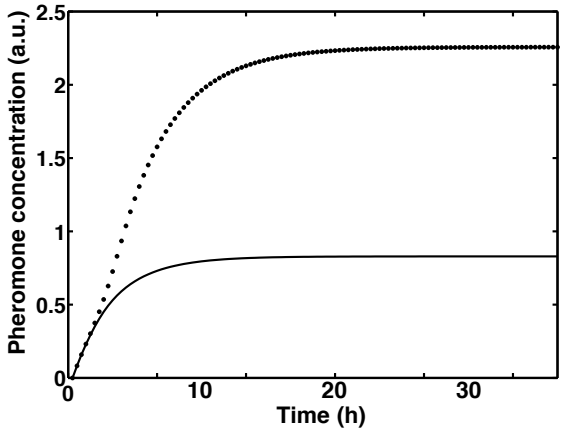

Figure 3.11: Concentration of pheromone a in the lawn for a standard avoidance experiment (continuous) and a conditioning (dotted) experiment when $k_{io}^R = k_{io}^T = 0$.

black - 16 worms). The model clearly reproduces the initial acceleration in the avoidance behavior but then the curves relax back to steady state levels (Fig. 3.6). This is a departure from the measurement where avoidance was monotonously increasing (especially in case of $N = 16$).

3.3 Conclusions

In the attempt to model the pathogen avoidance behavior we proposed two models that were able to reasonably reproduce major features of the data at two extremes. The empirical model relating the bacterial lawn spreading and avoidance was successful at later times in the experiment whereas the phenomenological model centered around the interaction of the worms with the putative pheromone worked well during early times. This suggests that the dynamics is really two fold and these two effects drive the behavior in separate time regimes.

The initial 10 hours are driven directly by the alarm pheromone. Our phenomenological model was based on the idea that the worms constitutively secreted an alarm pheromone which they could interact with in a concentration dependent way. Although we managed to find a set of parameters for which we were able to reproduce some of the N dependence of the initial plateau (Fig. 3.6) there

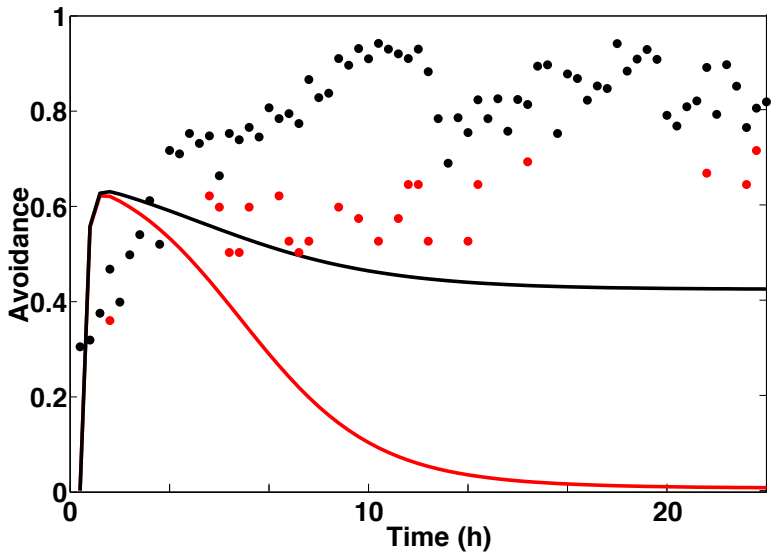

Figure 3.12: Accelerated avoidance curve (continuous curves) from the phenomenological model (Fig. 3.5) with the initial concentration of pheromone set at $a(t = 0) = 2.3$ to simulate a conditioned lawn (red - solitary worms, black - 16 worms). The dotted curves are data points from 30h conditioning experiment (Fig. 2.6) for comparison.

are still some notable departures (curves for $N = 4, 8, 16$) and the insensitivity of the model to three parameters (k^R_{io}, k^T_{oi} and j_T) points to a conclusion that some states or transitions may be redundant.

A model that combined both the effects of the pheromone and the spreading lawn would be a significant improvement. Such model would probably contain two sources of nonlinearity. Apart from the pheromone Hills function described above, it would also have a term that relates avoidance to bacterial spreading. As initially the driving force behind spreading is increased avoidance but later on it is probably the external bacteria that keep some of the worms outside of the original lawn the model would have to capture the feedback between these two effects.

3.4 Methods

The curves in Fig. 3.1ABC and Fig. 3.2 were fitted using Matlab Open Curve Fitting app (`cftool`) with custom fit equations and default settings.

The best parameters for the model in Eq. 3.2-3.7 were found using a nonlinear least squares solver (`lsqcurvefit`) with trust-region-reflective algorithm, maximum number of evaluations and iterations both set to 1000 and lower and upper bounds for each parameter defined as $(0,\infty)$. We used only first 40 frames (13h) of each avoidance curves. The set of parameters that we used throughout this chapter to plot the model results in Fig. 3.6-3.12 were the ones fitted to $N = 12$ avoidance curve: $k_i^{RT} = 134.1493$, $k_{io}^{T} = 1.1499$, $k_{oi}^{T} = 2.9534$, $k_o^{TR} = 0.9198$, $k_{oi}^{R} = 0.1437$, $k_{io}^{R} = 0.0049$, $j_T = 0.0023$, $j_R = 0.0011$, $\lambda = 0.0166$, $n = 2.1149$ and $a_0 = 21.5474$. The initial conditions used were $N_i^T = N_o^T = N_o^R = 0$, $N_i^R = 12$ and $a(t = 0) = 0$.

The curves predicted by the phenomenological model were found using an ordinary differential equation solver `ode45` using above parameters and appropriate initial conditions.

4

Molecular mechanics of collective avoidance

Once we established that pathogen avoidance in *C. elegans* depends on the nematode density we decided to take advantage of the accessible genetics of this model organism and investigate the molecular mechanism behind this phenomenon. We hypothesized that the interaction between conspecifics is mediated by a secreted chemical (i.e. the alarm pheromone) and therefore we will look for neurons that are necessary in detecting it (section 4.1) and also test whether any known genes involved in pheromone biosynthesis are also required in its production (section 4.2). Then we will test whether

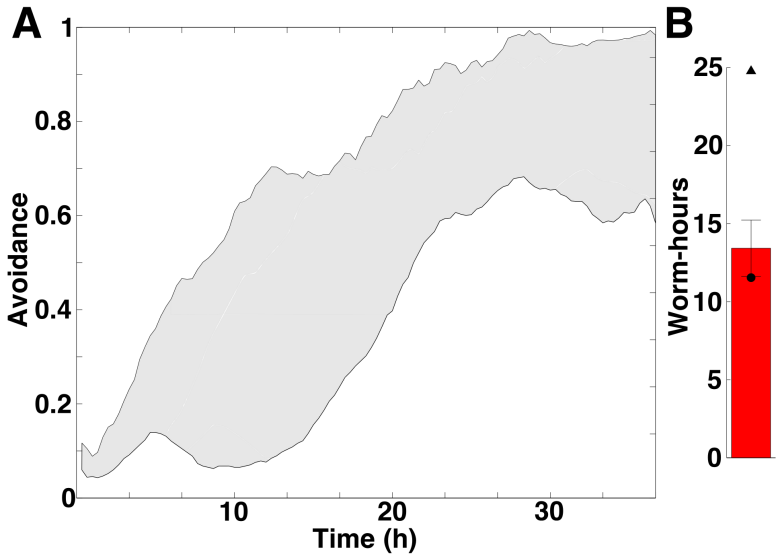

Figure 4.1: (A) shows averaged avoidance curves for solitary and multiple N2 worms. Shaded area between them is a measure of collective avoidance and corresponds to the red bar in (B). (B) The triangle (circle) shows the area under a multiple-worm (solitary) avoidance curve in (A), the red bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

serotonin (crucial in modulating aversive learning[245]) or indeed any other bioamine is necessary for collective avoidance regulation (section 4.3). Finally, we will ask whether this behavioral defense mechanism is dependent on any innate immunity genetic signaling pathways that help *C. elegans* survive infection by PA14 (section 4.4).

For brevity, instead of presenting the full avoidance curves for multiple and solitary worms, we will compute a difference between them. Each experiment produces several avoidance curves for a given condition $a_i^N(t_j)$, where i is a repeat number ($i = 1, 2, \ldots, r$), N is the condition, e.g. a number of worms and j is the frame number. $a_i^N(t_j)$ is just a fraction of worms outside of the bacterial lawn - $0 \leq a_i^N(t_j) \leq 1$ at each time point t_j ($t_{j+1} - t_j = 20min$). The total area under the avoidance curve of one of the repeats is just a sum of all the avoidance fractions:

$$A_i^N = \sum_{j=1}^{j_{max}} a_i^N(t_j).$$

Then we can find the average area under the avoidance curves for multiple worms ($\mathcal{A}^{16}$ - a triangle in Fig. 4.1B) and solitary worms ($\mathcal{A}^{1}$ - a full circle in Fig. 4.1B) and plot the difference between them as a bar graph (Fig. 4.1B) with the error bar corresponding to the added standard deviations for each condition. Fig. 4.1A shows an example of two average avoidance curves for solitary and 16 worm populations of *C. elegans* N2 (wild type strain) and the shaded area between them corresponds to the red bar in Fig. 4.1B.

4.1 Neurobiology of *C. elegans*

C. elegans has a simple but non-trivial neural system that enables it to detect a wide variety of chemical compounds[13]. Therefore, if the collective avoidance is driven by a chemical secreted into the bacterial lawn it has to be then detected by other nematodes. We therefore expect that one or more of the worm's sensory neurons will carry the receptor that binds the alarm pheromone and drives the communication between the nematodes in the avoidance experiment. In this section we will identify this neuron.

4.1.1 Background

C. elegans hermaphrodite has 302 neurons that fall into three main categories[13]: motor neurons (which produce muscle contractions), interneurons (integrate sensory signals and relay commands to motor neurons) and sensory neurons (which gather information from external as well as internal environments). The division is not clear cut as some of these neuronal cells may fall in more than one category.

Apart from mechanosensory neurons[82] (responsible for sensing touch) and O_2/CO_2 sensors[34,246,30] the majority of sensory neurons are located in two pairs of bilateral organs (Fig. 4.2a) - amphids[6] (located in the anterior of the pharynx[4]) and phasmids[86] (in the posterior of the animal).

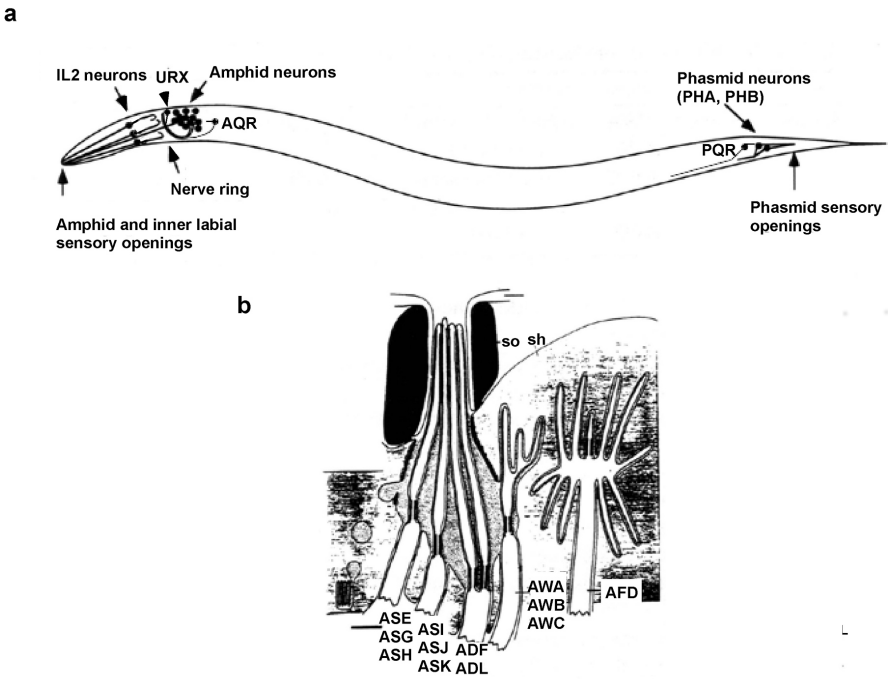

Figure 4.2: (A) Shows a schematic of *C. elegans* with two sensory organs: amphid and phasmid in the anterior and posterior of the animal. The cilia that detect chemical cues from the environment in the amphid extend back towards the nerve ring where bodies of the nerve cells are located and where they make synaptic connections to other neurons. (B) Shows a schematic cross section through the amphid with seven ciliated neurons in direct contact with the environment and four neurons whose cilia terminate in the cuticle. [13]

Amphids and phasmids provide openings for neuronal cilia (thin microtubule based extensions specialized as receptors[187]) to make contact with the external environment. Amphids contain 12 pairs of sensory neurons (3 pairs in phasmids). Eight pairs are directly exposed[160] and thought to be receptors for water-soluble chemicals, the remaining neuronal endings are covered by a sheath cell and are not exposed through the amphid pore (Fig. 4.2b). Of the latter neurons, three (AWA and AWC thought to detect attractive and AWB repulsive stimuli[226]) are olfactory receptors[16] and AFD has been primarily studied for its role in thermal sensation[150].

The sensory neurons are able to detect environmental chemical cues thanks to G protein-coupled receptors (GPCRs)[13]. These proteins are localized to a cell membrane (usually in the cilia in the sensory neurons[223,57]) and contain an extracellular part which binds to a specific ligand upon which a conformation change alters its cytoplasmic part's interaction with a G-protein[158,170,171]. The G-proteins then are able to transmit the environmental signals downstream[73]. *C. elegans* genome contains a strikingly abundant and diverse collection of putative GPCRs[179]. Well over a thousand genes (7% of the entire genome) in its DNA are candidate receptors which could be classified in 19 families - a much higher GPCR genetic variability than in mammals or insects[219]. However, a systematic knock down study[112] has shown that few GPCRs, if any, are essential genes for *C. elegans* viability. Furthermore, because GPCRs are far more numerous than individual sensory neurons, many different receptors are expressed in each cell, some of them may have redundant or overlapping functions (e.g. in the type or a concentration range of a ligand they bind). This makes it difficult to determine ligand-GPCR pairs and so far only a handful receptors have been unambiguously paired with their ligands[192,244].

4.1.2 Sensory neurons involved in collective pathogen avoidance

To test whether any of the ciliated neurons is involved in the collective avoidance we tested *osm-6(p811)* mutant, which is defective in development and sensory function of all ciliated chemosensory

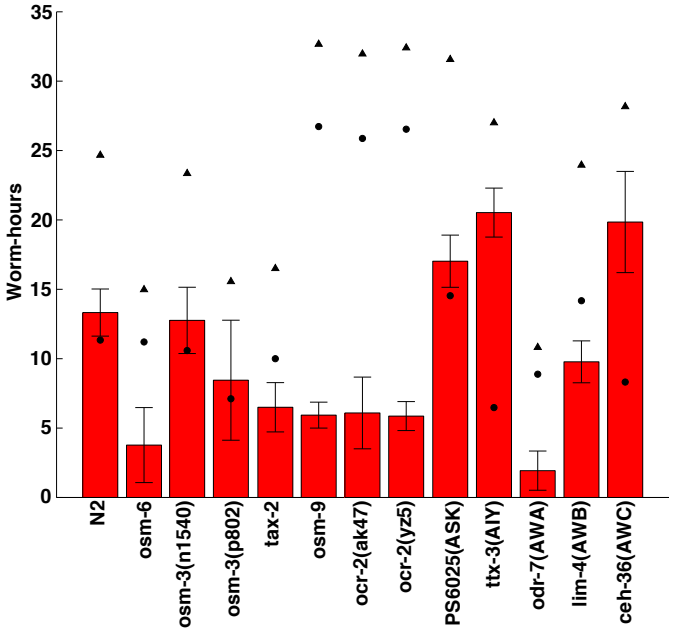

Figure 4.3: Collective avoidance for various neuronal mutants. The triangle (circle) shows the area under a multiple-worm (solitary) avoidance curve, the red bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

neurons[45]. The 16-worm avoidance curve was delayed and single worm plates were similar to N2 (Fig. 4.3) suggesting that ciliated sensory neurons are involved in this behavior.

To narrow down the set of neurons that are relevant for this phenotype we tested an *osm-3(p802)* mutant, which is defective in function of all chemosensory neurons except olfactory neurons (i.e. AWA, AWB and AWC)[213] as well as another allele - *osm-3(n1540)*, which is defective in all chemosensory neurons apart from olfactory and ADF[194]. Fig. 4.3 shows that the difference between single and 16 worms in *p802* mutant is diminished but *n1540* looks almost like N2 indicating that the set of sensory neurons necessary for the density dependent avoidance behavior is a subset of AWA, AWB, AWC and ADF.

We can further divide the sensory neurons into two groups depending on a type of ion channel that they express. Four chemosensory neurons express *ocr-2/osm-9* - a transient receptor potential

(TRPV) channel[221] and the rest a *tax-2/tax-4* - a cyclic nucleotide-gated channel[125,43] (table 4.1). In AWC and ASE *osm-9* functions additionally as a regulator[13].

Table 4.1: Amphid sensory neurons that express two different two-component calcium channels: TRPV channel (*ocr-2/osm-9*) and cyclic nucleotide-gated channel *tax-2/tax-4*.

ocr-2/osm-9	AWA	ADF	ASH	ADL			
tax-2/tax-4	AWB	AWC	ASE	ASI	ASG	ASJ	ASK

tax-2 mutation has reduced the difference in avoidance between single and 16 worms suggesting that this calcium channel is involved in the collective avoidance. *osm-9* mutant[221] had a very different effect - although it did reduce the difference between a single and 16 worm curves it also accelerated the entire avoidance dynamics (note the areas under avoidance curves for multiple and solitary worms is much higher than for N2). Eliminating the second component of the TRPV channel - *ocr-2* produced the same effect[221,197].

We can further narrow down the search for minimal subset of relevant neurons by testing genes that are terminal differentiators of specific neurons. *ttx-3*[8], *lim-4*[183] and *ceh-36*[129] are responsible for specification of AIY interneuron, AWB and AWC sensory neurons respectively. Mutations in those genes do not significantly disrupt the community dependent avoidance behavior indicating that those neurons are either redundant or do not take part in this phenotype (although they were all implicated in aversive learning[245,85] or pathogen avoidance[163] in the past). Detailed avoidance curves for AWB and AWC are shown in Fig. 4.4. Similarly, ASK neuron appears to be unnecessary as the transgenic worm PS6025 (expressing a *qrIs2 [sra-9::mCasp1]* construct that genetically ablates the cell) still is capable of collective avoidance.

The last remaining olfactory neuron presented a different story. Mutation in *odr-7* required for AWA specification[193,192] eliminated the difference between single and multiple animals by delaying the 16 worm curves (Fig. 4.5). This brought down the collective avoidance to levels of *osm-6* (Fig.

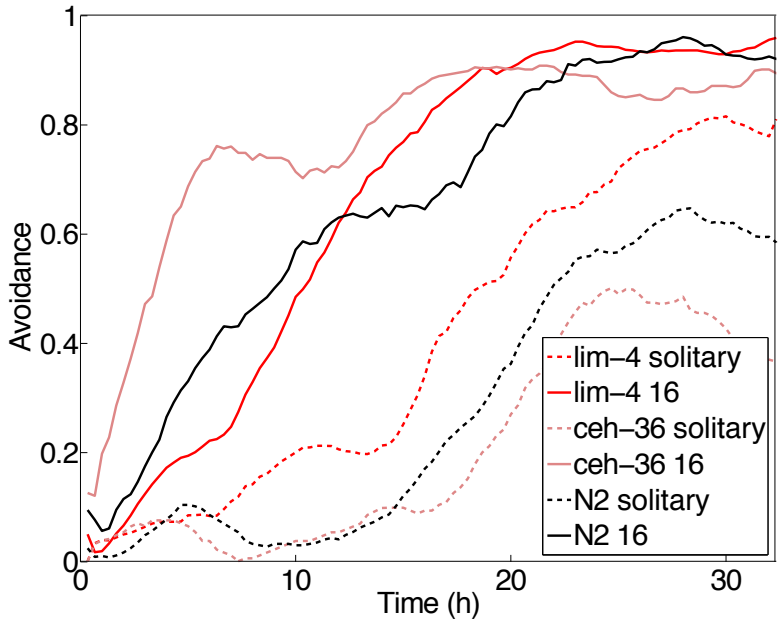

Figure 4.4: Pathogen avoidance of solitary and groups of 16 *lim-4* and *ceh-36* mutants deficient in AWB and AWC neuron development respectively.

4.3) indicating that AWA neuron is essential for this behavior.

To verify that *odr-7* worms were unable to detect signals that promote collective avoidance but were capable of producing the pheromone we performed conditioning experiments with *odr-7* and wild type animals. First, we conditioned a lawn of PA14 with *odr-7* animals for 18 hours with a metal ring that prevented them from leaving the lawn. We then removed the rings and replaced the mutants with naive wild type worms. These nematodes avoided the conditioned lawns with similar kinetics to avoidance on lawns preconditioned by other wild type worms suggesting that *odr-7* mutants are capable of producing the avoidance signal. In turn, *odr-7* animals exposed to lawns conditioned by wild type worms for 18 hours stayed in the lawn for much longer than wild type worms (Fig. 4.6). This result supports a hypothesis that *odr-7* animals are defective in detection of the alarm pheromone, but are adept at its production.

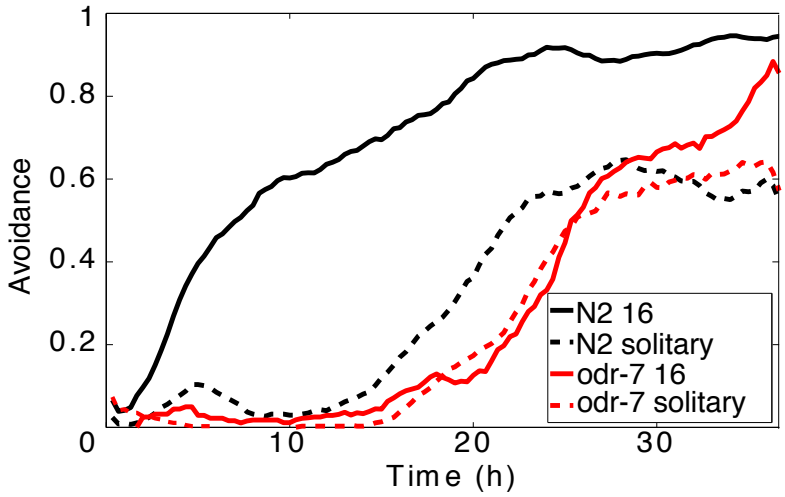

Figure 4.5: Pathogen avoidance of solitary and groups of 16 *odr-7*mutant deficient in AWA neuron development.

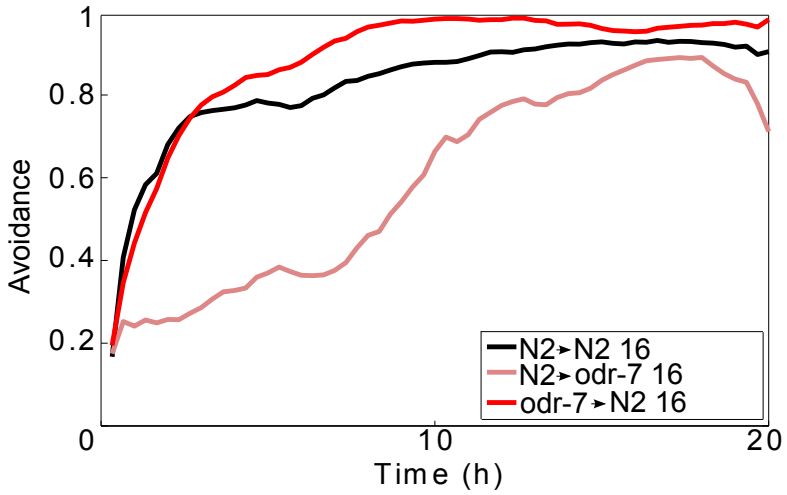

Figure 4.6: Avoidance of preconditioned plates. Wild-type worms avoided lawns that were preconditioned by *odr-7* mutants, but not the other way around.

4.2 Pheromones and conspecific communication

A systematic research on the pheromonal communication between *C. elegans* has started only 10 years ago[109,135] and we know only a small fraction of the genetic network involved in the biosynthesis of those molecules. Nevertheless, in this section we will ask whether any genes necessary for pheromone production are also required for the generation of the putative alarm pheromone and therefore necessary for collective avoidance?

4.2.1 Background

Apart from behaviors that *C. elegans* are capable of executing by themselves they can also perform actions in coordination with their conspecifics. Collective behaviors in *C. elegans* have been known for over three decades. The developmental decision of whether to enter the alternative larval stage *dauer* was found to be regulated by the concentration of constitutively expressed pheromones (daumone), which the worms use as a proxy to measure population density[80]. However, the chemical nature of the pheromones remained elusive until 2005 when[109] they were shown to be a cocktail of several different fatty acid derivatives, known as ascarosides[135].

Ascarosides were originally purified from a layer of an egg of a human intestinal parasite *Ascaris lumbricoides*[71] over a century ago but it took additional four decades for a detailed chemical analysis[67,72]. Common structural features of these signaling molecules[58,200] include the ascarylose core with a fatty acid derived side chain (Fig.4.7). In addition, different ascarosides have one or more residues that are derivatives of tryptophan, glucose or p-aminobenzoic acid (PABA) that are attached to the asacrylose ring or at the end of the side chain leading to a large combinatorial diversity. Hundreds of different ascarosides have been isolated and were shown that their function changes depending on concentration and presence of other ascarosides in the mix[230].

Recent work[200,230] has determined that the ascaroside side chain undergoes a peroxisomal β-

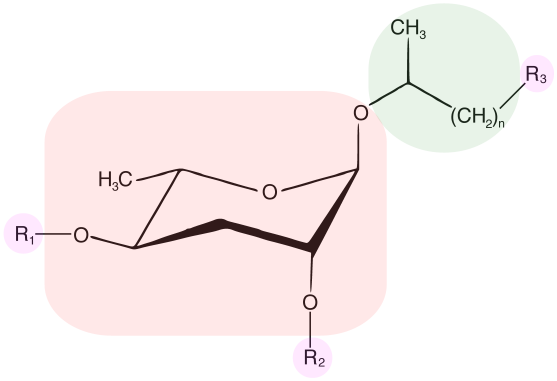

Figure 4.7: A schematic representation of ascaroside structure. The molecules are composed of the asacrylose ring (red), a fatty acid derived side chain (green) and several residues that are derivatives of tryptophan, glucose or p-aminobenzoic acid (magenta) [200].

oxidation - a 4-step process that normally shortens long-chain fatty acids iteratively by two carbon units, producing acetyl-CoA that enters the citric acid cycle [135,172]. The first gene *daf-22* involved in this bioreaction was identified shortly after discovery of the daumone by random mutagenesis and systematic screening for mutants unable to produce the dauer pheromone [81]. Interestingly, transgenic intestinal *daf-22* expression was later found to be sufficient to restore dauer activity of *daf-22* mutants [24], suggesting that ascarosides are synthesized in the intestine. More recent mass spectrometry-based comparative metabolomics revealed three other genes involved in the process: *acox-1* [111], *maoc-1* [230] and *dhs-28* [26].

The generation of ascaroside side chain is the best studied aspect of the pheromone biosynthesis process (Fig. 4.8). Ascarylose biosynthesis on the other hand, has not been investigated in detail [135]. There are a few candidate genes that are homologous to bacterial ascarolyse (e.g. *ascE* from *Yersinia pseudotuberculosis* [230,220]) but they have not been tested. Similarly, genes required for attachment of different residues are unknown.

More recently, ascaroside synthesis and excretion was shown to be dependent on a number of environmental factors. The concentration of different pheromones inside the animal (and outside

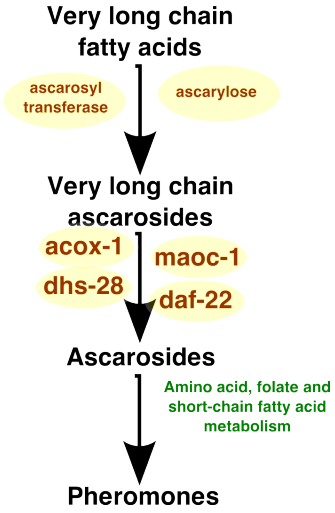

Figure 4.8: A putative model of ascaroside synthesis from very long chain fatty acids that are processed in the peroxisomes and upon addition of ascarylose are transformed into ascarosides whose side chains are then trimmed by sequence of different enzymes including *acox-1*, *maoc-1*, *dhs-28* and *daf-22*. Finally, different functional groups are added to the molecules that specify their functionality[230].

in a liquid media) depends not only on the sex of the animal[106], but also its diet and life stage[115], temperature of cultivation[25,111,131] and whether the animals are well fed or starved[230]. Therefore, it should not come as a surprise that ascarosides could be exploited by the nematodes to communicate their status to their conspecifics and drive more complex behaviors this way[58].

Apart from the decision to enter an alternative developmental stage dauer[80], daumone has been implicated in regulating the population density dependence of chemotaxis[142] and olfactory plasticity[237], i.e. change in chemotactic response due to experience. Other ascarosides were shown to control mating behaviors[199] as well as aggregation and repulsion[137,200,114] and even longevity and stress resistance[136].

This list is non exhaustive as many more population density dependent behaviors were already reported but no obvious pheromone link was identified yet. In worm populations at higher densities nematodes are more likely to disperse to search for other patches of food[93,79], starving larvae are more likely to survive for longer[10] and large populations drive a more robust development of

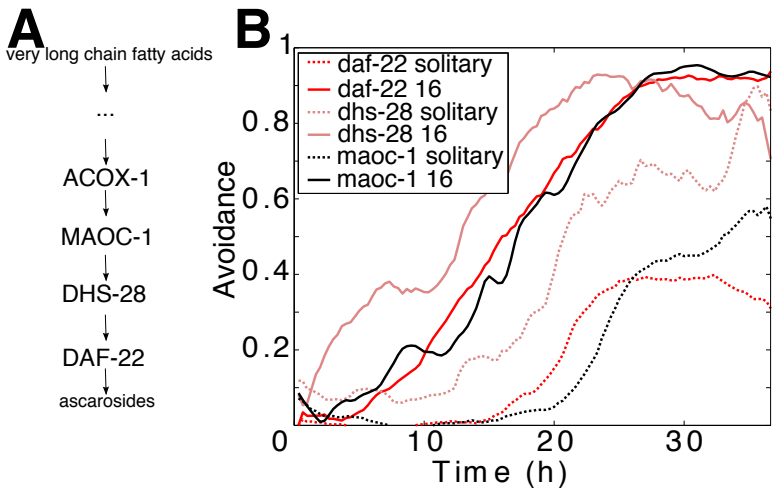

Figure 4.9: (A) Pheromone biosynthesis pathway[230]. (B) PA14 avoidance of solitary and groups of 16 mutant worms deficient in pheromone synthesis genes.

neurons[181].

4.2.2 Pheromone biosynthesis network is involved in avoidance signal production

To test if the putative alarm pheromone was related to ascaroside biosynthesis we characterized the avoidance dynamics in worms carrying loss-of-function alleles for each of the four genes encoding the enzymes that catalyze ascarosides, *acox-1(ok2257)*[111], *maoc-1(hj13)*[230], *dhs-28(hj8)*[26] and *daf-22(m130)*[81] (Fig. 4.9A). Loss of each of the three downstream genes did not eliminate the difference between solitary and 16 worms (Fig. 4.9B), although overall avoidance was sometimes delayed. In contrast, both solitary worms and groups of 16 *acox-1* animals avoided PA14 with the same kinetics as solitary wild-type (N2) worms, failing to display the community effect (Fig. 4.10). These data suggest that *acox-1* is necessary for collective avoidance.

ACOX-1 (an ortholog of human acyl-CoA oxidase) is involved in fatty acid metabolism in *C. elegans* and its disruption has pleiotropic effects[240]. To verify that the lack of collective behavior in

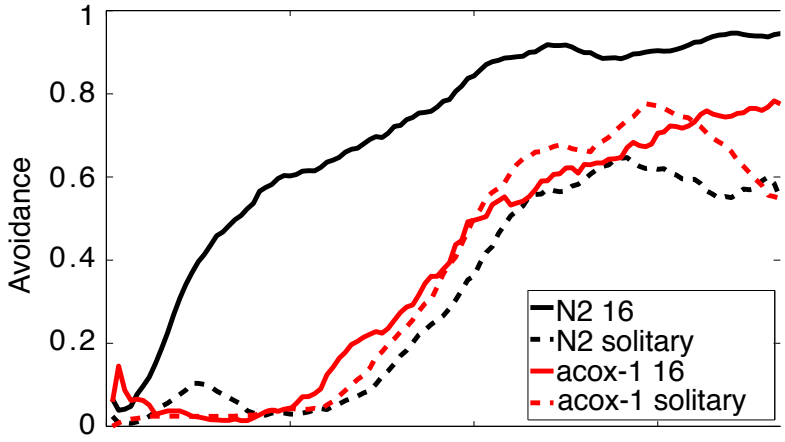

Figure 4.10: Pathogen avoidance of solitary and groups of 16 *acox-1* mutant.

acox-1 animals was due to inability to produce a relevant pheromone rather than a sensory or physiological defect we conducted two conditioning experiments. First, we conditioned a lawn of PA14 with wild type animals for 18 hours with a metal ring that prevented them from leaving the lawn. We then removed the rings and replaced the wild type worms with naive *acox-1* mutants. Within 3 hours almost 90% of *acox-1* worms (both solitary and in groups) left the lawn, suggesting that they were able to detect the signal left there by the wild-type worms (Fig. 4.11). In contrast, wild type worms exposed to lawns conditioned by *acox-1* animals for 18 hours stayed in the lawn for much longer than in lawns conditioned by wild type worms, and almost as long as in unconditioned lawns (Fig. 4.11). This result supports a hypothesis that *acox-1* animals are defective in production of the alarm pheromone, but are adept at its detection.

4.3 Endocrine system

Bioamines are involved in regulation of virtually all behaviors in *C. elegans*[36] and therefore it is natural to ask what is their effect on collective avoidance? We investigated this question by performing avoidance experiments on mutant *C. elegans* in genes encoding enzymes involved in synthesis or

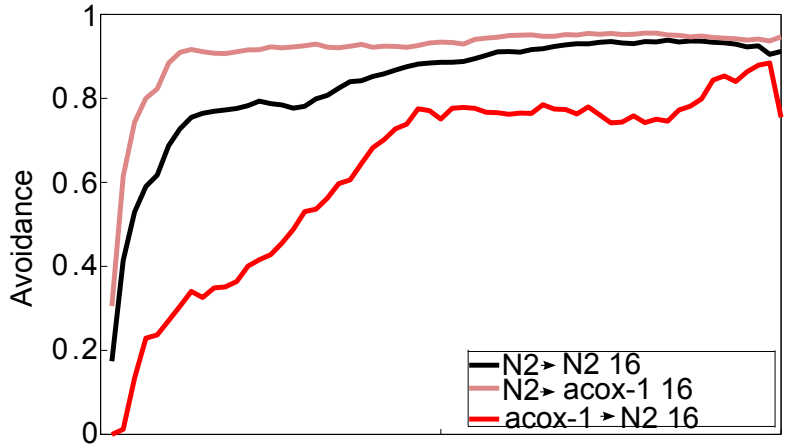

Figure 4.11: Avoidance of preconditioned plates. Wild-type (N2) worms were placed on lawns that were preconditioned by *acox-1* mutants for 18 hours (red curves). Their avoidance was not significantly accelerated. Conversely, when N2 worms were used in the preconditioning stage and the avoidance of *acox-1* worms was recorded the avoidance was accelerated (brown curve).

transport of these molecules and receptors through which bioamines effect their function.

4.3.1 Background

Most of the *C. elegans* behaviors are modulated by four biogenic amines: octopamine, tyramine, dopamine and serotonin[36]. They were detected in the animals using high performance liquid chromatography[102,205] and have been hypothesized to function as neurotransmitters. Other bioamines that are present in mammals (histamine, epinephrine, and norepinephrine) were not found in these nematodes[102]. Dopamine and serotonin have established roles in human nervous system and deficiencies in signaling by these neurotransmitters has been linked to many human diseases making them an attractive research topic in this model organism[36].

Dopamine (DA) and serotonin (5HT) are synthesized in two step processes starting from tryptophan and tyrosine amino acids respectively (Fig. 4.12). The first step is rate limiting for both of them and involves tryptophan (tyrosine) hydroxylase encoded in the gene *tph-1*[212] (*cat-2*[133]) and a *tph-1* (*cat-2*) knock out animals are unable to produce endogenous serotonin (dopamine). The second

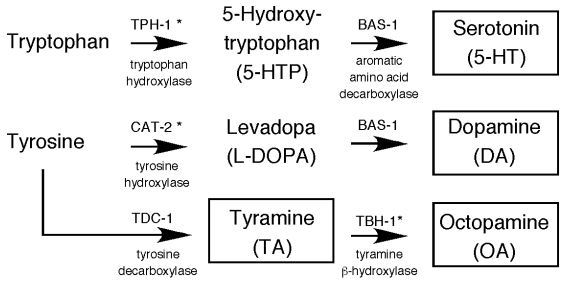

Figure 4.12: *C. elegans* uses four bioamines as neuropeptides that modulate all of its behaviors. Serotonin, dopamine, octopamine and tyramine are all derived from tryptophan and tyrosine amino acids through a series of steps [36].

step is common to both amines and requires an aromatic amino acid decarboxylase encoded in *bas-1* gene [134].

In turn, tyramine (TA) and octopamine (OA) are synthesized sequentially (Fig. 4.12). First, tyrosine decarboxylase TDC-1 turns the amino acid into tyramine and then tyramine β-hydroxylase TBH-1 transforms it into octopamine [5]. Once the amines are synthesized they are deposited in a vesicle and transported outside the cell into a synapse using a vesicular monoamine transporters encoded in *cat-1* gene [55].

Among almost a 1000 somatic cells in *C. elegans* only a handful are capable of synthesizing bioamines. Using antibody staining it was found that tyramine is present at very low concentrations (perhaps because it is used as a substrate for octopamine) and both TA and OA are restricted to only a few cells [5]. Nevertheless, they were found to be functional through a number of different receptors. *octr-1* [235] and *ser-3* [209] for OA and *ser-2* [176,177] and *tyra-2* [175] for TA.

Dopamine is produced in eight mechanosensory neurons in a hermaphrodite [205] and ablation of these cells or a mutation that stops the synthesis of the amine impairs the animal's ability to sense or respond to changes in its environment [184,19]. For example, the physical sensation of bacterial food stimulates these cells to release DA [119,113] which in turn affects a range of behaviors: locomotion rate [185], mating [134], foraging [95], response to soluble repellants [65] and adaptation to a stimulant after a prolonged exposure [44]. Similarly to octopamine and tyramine, dopamine modulates these behaviors

through a set of at least four receptors[159] *dop-1*[210,184], *dop-2*, *dop-3*[37], *dop-4*[204,211].

Serotonin is produced in six types of hermaphrodite neurons: ADF, HSN, NSM, AIM, VC4,5 and RIH[212,185]. The most interesting of these is a pair of ADF neurons - one of 12 sensory neurons located in the amphid.

The basal expression level of *tph-1* in ADF neuron is turned on by OCR-2/OSM-9 TRPV channel (signal transduction calcium channel present in several other sensory neurons[221]) under optimal growth conditions[241]. Exposure of *C. elegans* to aversive conditions (including pathogenic bacteria[245]) increases ADF activity[166] which leads to upregulation of *tph-1*[151] in a *unc-43* dependent way[173,180,178,228].

This upregulation in 5HT when exposed to a pathogen is driven by the same TIR-1-SEK-1-NSY-1 pathway that is simultaneously responsible for resistance to heavy metals[127], immune response in the epidermis and the intestine (through *pmk-1* transcription factor)[195] and a development of some neurons[41]. Mutation in this pathway leads to a severe increase in *C. elegans* susceptibility to PA14 pathogen[164,132,195]. Transgenic rescue of this pathway in the intestine, restores the resistance almost to the wild type levels and the remaining difference in survival has been explained by the action of the pathway in ADF neuron where it drives the pathogen avoidance behavior[195]. TIR-1-SEK-1-NSY-1 pathway in ADF up regulates *tph-1* (through *atf-7*/*daf-19* transcription factors[236]) in the neuron, which in turn leads the worms to physically avoid the pathogen. A more limited exposure results in slight improvement in survival. This explanation of the effect of 5HT increase on survival remains contentious as two other studies did not observe this small effect[245,116].

There are five confirmed serotonin receptors in *C. elegans*[29,89]: a serotonin gated chloride channel *mod-1*[168] and four GPCRs: *ser-1*[87], *ser-4*[155], *ser-5*[89] and *ser-7*[98]. In addition there is also a serotonin reuptake channel *mod-5*[169] which absorbs excess 5HT from a synapse back into the neuron. All of these receptors are expressed in various neurons[168,91,89,53,107] and in some muscles[227,97,29]. Interestingly, many of *C. elegans* 5HT receptors are expressed on neurons that are not directly innervated

by serotonergic neurons, which suggests that most serotonergic signaling is humoral and extra-synaptic[168,169,53].

Various 5HT receptors mediate different phenotypes that serotonin was implicated in. *mod-1* and *ser-1* are involved in food-induced slowing[168,53]. *ser-7*, *ser-4* and *ser-1* connect 5HT to its effects on increased egg-laying[98,28,52,126] and accelerated pharyngeal pumping[98]. *ser-5* takes part in stimulating aversive responses on food[89].

In PA14-*C. elegans* system the role of various serotonin receptors was not widely studied. Only *mod-1* has been tested and found to mediate the aversive learning phenotype[245,85]. The effect of other 5HT receptors on aversive learning, avoidance or survival on PA14 has not been reported.

4.3.2 Endocrine system influence on collective avoidance

A vesicular monoamine transporter CAT-1[55] shuttles the bioamines to extracellualr space. A *cat-1* mutant shows a very similar density-dependent avoidance as a wild type animal (Fig. 4.13). This suggests that bioamines either perform a function within the cells where they were produced or they are shuttled outside using other means for the purposes of collective avoidance.

Next, we investigated the function of serotonin which had been identified as playing an important role in aversive learning[245] and avoidance[195]. As expected, the difference between multiple and single *tph-1* worms was significantly reduced, but not eliminated (Fig. 4.13). In the *acox-1* and *odr-7* cases, multiple worm avoidance was delayed to match the single worms, allowing for straightforward interpretation. However, in the *tph-1* case both population densities were affected: avoidance of solitary worms was accelerated and that of multiple ones delayed. Furthermore, all the serotonin receptors we tested resulted in the opposite effect, namely the difference between avoidance of solitary and multiple worms increased(Fig. 4.13, green columns). We confirmed it further when we tested a quintuple mutant *ser-1;ser-4;ser-5;ser-7;mod-1*[88] that was missing all the known serotonin receptors.

Apart from serotonin receptors we also tested *mod-5* which is a 5HT reuptake channel which

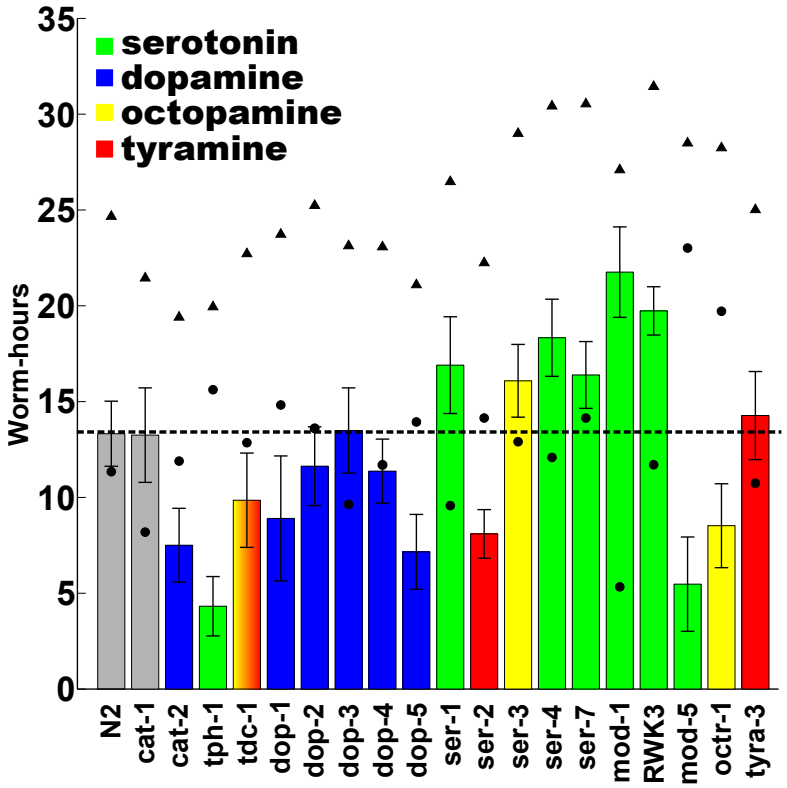

Figure 4.13: Collective avoidance for various endocrine mutants. Blue, green, yellow and red bars correspond to genes involved in dopamine, serotonin, octopamine and tyramine regulation respectively. A vesicular monoamine transporter CAT-1 is used by all four bioamines for transport out of the cell and TDC-1 is involved in synthesis of both octopamine and tyramine. The triangle (circle) shows the area under a multiple-worm (solitary) avoidance curve, the bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

shuttles excess extracellular serotonin back into cells[169]. Although, this mutant successfully reproduced the difference between solitary and multiple worm avoidance of *tph-1* it also significantly accelerated avoidance overall.

Octopamine and tyramine biosynthesis requires a step involving *tdc-1*[5] gene. A *tdc-1* mutant shows a modest reduction in collective avoidance (Fig. 4.13) but, similarly to serotonin, this effect is not reproduced by a deletion in a single receptor mutant.

Finally, the last dopamine mutant - *cat-2* shows a reduction in collective avoidance (Fig. 4.13). Furthermore, out of five dopamine receptors only two of them (*dop-1* and *dop-5*) partially reproduce the dopamine-deficient mutation and the remaining three do no significantly differ from the wild type.

This suggests that in contrast to neurons and pheromone biosynthesis, the role of bioamines is significantly more complex. Each bioamine seems to be affecting differentially avoidance of both solitary and multiple worms and their respective receptors not only do not necessarily reproduce the bioamine mutants but often have the opposite effect (as is the case with serotonin). This perhaps should not be surprising given that each of these molecules takes part in a wide array of different behaviors, often exerting opposite effects through different receptors[88] or modulating activity of other bioamines[185]. Furthermore, pathogen avoidance itself is a complex behavior that involves several balancing forces (hunger and perception of danger) that drive the animals in opposite directions which suggests that a more sophisticated approach is needed to decipher the role of different bioamines in this phenomenon.

4.4 Innate immune system

Behavioral avoidance and innate immunity are two major ways in which *C. elegans* deal with pathogenic bacteria in their environment. In principle it could be beneficial for the animal if these two re-

sponses were differentially regulated. For example, if the worm was able to determine that a pathogen was too dangerous it would avoid the exposure and risk going hungry rather than feed on the bacterium at the risk of dying before it is able to reproduce. Additionally, it has already been noted that similar pathways acting in different tissues are responsible for both immune and behavioral responses[222,242,1]. A question arises, do immune signaling pathways affect collective behavior in *C. elegans* ?

4.4.1 Background

Apart from behavioral avoidance, *C. elegans* has two other major defenses against microbial attacks[60]. The first one is a physical barrier between the environment and the organism - a strong cuticle, made of collagen and chitin which make up the exoskeleton of the worm. In addition, the pharyngeal grinder destroys microorganisms that are taken up during feeding. This prevents live pathogens from reaching the intestine and establishing an infection. In fact, mutants that have a defective grinder are found to be more susceptible to bacteria[196].

The second line of defense is the cellular immune system. *C. elegans* , just like other invertebrates, lacks a mammalian-style adaptive immune system. Furthermore, unlike other invertebrates, the nematodes do not have specialized immune cells similar to, for example, Drosophila where macrophage-like hemocytes, can engulf invading microbes (the only cells in the *C. elegans* body cavity are six coelomocytes which are incapable of phagocytosis but rather function as scavenger cells with high endocytic capacity)[69]. Despite these differences *C. elegans* has a complex inducible defense mechanism that involves multiple signaling cascades which regulate the production of antimicrobial peptides and proteins (e.g. caenopores[27], lysozymes[188] and lectins[190]) with a proven record of antibacterial activity[104] in a pathogen- and tissue-specific way[60].

A model of pathogenesis of *C. elegans* has been proposed 15 years ago[215] and within this short amount of time a great complexity of the nematode's immune system has been described[117,141,61]. It is

unsurprising that a bacterivore that in the wild has to interact with bacterial[216], fungal[108] and viral[75] pathogens has evolved a variety of genetic strategies of protecting itself. The microbial attacks must usually overcome the physical barriers first before the infection can take place[48]. Much research has been focused on bacteria that are able to avoid being destroyed in the grinder and establish intestinal colonies[105,191]. Other bacteria use anal[99] and uterine[152] openings to invade the nematode. Some fungal pathogens attach themselves to the cuticle and grow into the animal (using amphid or vulval orificies)[108]. Although all of these pathogens stay out of the cell of *C. elegans* , this is not a rule as the first intracellular fungal pathogen was discovered recently[225]. Finally, some microorganisms do not have to physically invade the animal to kill it. The plague bacteria (*Yersinia pestis*) creates a film in front of *C. elegans* pharynx and blocks it's food intake[49] and various *Pseudomonas* produce toxins that under the right conditions lead to the nematode's quick death[138,77].

One of the first models[215] of *C. elegans* pathogen infection used *Pseudomonas aeruginosa* PA14 - a gram negative bacterium which is an opportunistic human pathogen. The strain PA14 was a human isolate[167] that was subsequently found to use an overlapping set of virulence factors during pathogenesis of plants, mice and *C. elegans*[216].

A killing assay protocol is relatively straightforward and consists of incubating the PA14 in liquid culture overnight in rich media, spotting it on low-osmotic media and incubating further for 24-48h and then exposing thusly prepared bacteria to a population of synchronized *C. elegans* young adults or L4 larvae. Because PA14 are the only source of food on the plate the nematodes are forced feed on them. Within the first 24h first nematodes die and the majority of them perish within the first 72h. By plotting a survival curve (a cumulative count of dead worms over time) one could distinguish between more or less lethal strains and mutations of PA14[70] thus identifying the virulence factors of the bacterium. One could also screen through randomly mutagenized *C. elegans* to identify genes that increase the susceptibility of the worm to the pathogen which would reveal pathways important in mounting an immune response[141].

One such screen revealed a presence of p38 mitogen-activated protein kinase (MAPK) pathway[118]. This ancient immune pathway exists in all animals and plants[60,117] and in *C. elegans* acts through a cascade of kinases SEK-1, NSY-1 and PMK-1 that activate the immune response in the intestine[195] and epidermis[164]. Upstream of this pathway is another protein with highly conserved resistance domain *tir-1*[132,46]. A mutation in any of those genes results in highly increased mortality of *C. elegans* on PA14.

Another highly conserved system that has a profound influence on *C. elegans* resistance to a pathogen[78] involves insulin signaling. *daf-2* insulin-like receptor inhibits the downstream transcription factor DAF-16 by phosphorylation through a kinase cascade and regulates metabolism, reproduction, development, lifespan and resistance to a variety of environmental stresses[234]. It turns out that DAF-2 and DAF-16 also regulate immunity independently of the other functions[63] and in parallel to p38 MAPK pathway[224].

Next, a transforming growth factor-β-related gene *dbl-1* belongs to a large regulatory cascade which, just like *daf-2-daf-16*, regulates numerous processes (mainly during development) as well as immune response against bacterial[139] and fungal[247] pathogens.

A bZIP transcription factor *zip-2*[62] regulates a more specialized immunity pathway. A detailed investigation of *C. elegans* response to one of the virulence factors of PA14 - Exotoxin A (which inactivates translation in nematode's cells) has shown that *zip-2* activates a wide range of antibacterial response[145,56].

A more recent direction in immunity research in *C. elegans* is the observation of a strong confluence of innate immunity and neural system[222,242,1]. Some of the above pathways have a parallel function in different tissues. The p38 MAPK pathway functions in the intestine, where it leads to activation of PMK-1 and a release of an array of antimicrobial factors, whereas the same cascade functions in the ADF sensory neuron to trigger the aversive learning behavior[195]. Another pathway like that involves a *fshr-1* GPCR that is expressed both in the intestine and neurons and is strongly

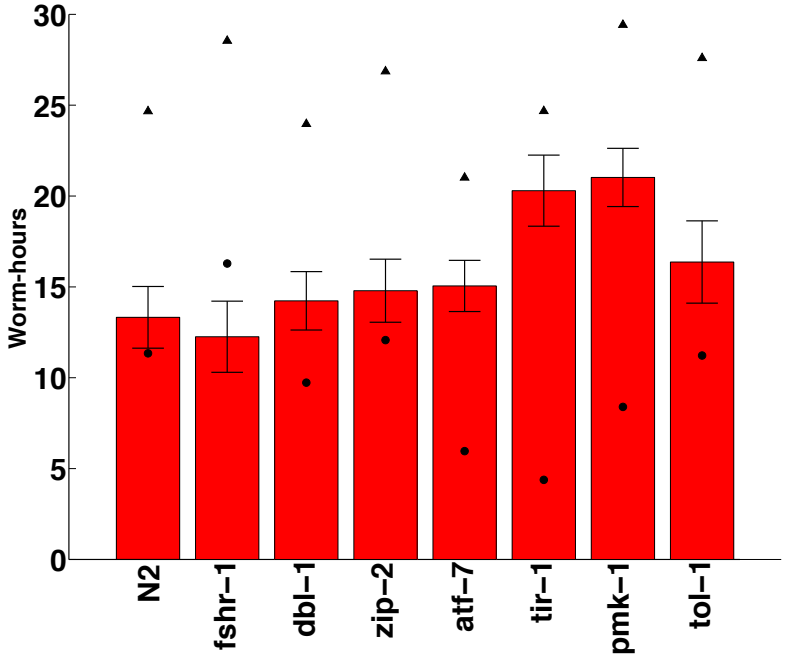

Figure 4.14: Collective avoidance for various immunity mutants. The triangle (circle) shows the area under a multiple-worm (solitary) avoidance curve, the red bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

influencing *C. elegans* susceptibility to PA14 but it's effect on behavior remains unknown[162]. Other pathways have been found to be directly acting through the neural system. The *daf-2* receptor on RIA interneuron is activated by insulin-like ligands produced in ASJ and ASI neurons[116,39] that leads to activation of behavioral and immune responses. In turn, the *dbl-1* immunity in epidermis is regulated by neurons as *dbl-1* neuropeptied is produced and released by certain neuronal cells[247] and is also required in aversive learning[243]. Finally, recent evidence suggests that *C. elegans* uses serotonin to downregulate its immune response to *M. nematophilum*[9] and a putative octopamine GPCR *octr-1* to enhance p38 MAPK response to PA14[208].

4.4.2 Innate immunity and collective avoidance

To answer whether innate immune system has any effect on collective avoidance we tested several mutants that were deficient in four different immune signaling pathways. *fshr-1*, *dbl-1* and *zip-2* are parts of three independent pathways that lead to an upregulation of antibacterial factors upon infection[162,139,62]. All three mutants show a similar level of collective avoidance as the wild type (Fig. 4.14) suggesting that there is no interaction between these two effects.

tir-1 contains a highly conserved resistance domain and activates a fourth and probably the most studied immunity pathway - p38 MAPK[132,46]. It has different roles in various tissues. In the ADF neuron it acts through *atf-7* transcription factor to upregulate the expression of serotonin and drive aversive learning[236]. In the intestine and epidermis, it signals through *atf-7* and *pmk-1* kinase to activate the immune response[195,164]. When all three mutants are exposed to PA14, they still respond differently depending on the population density, but in a way distinct from the wild type leading us to a conclusion that the putative coregulation of collective avoidance and the immune system is more subtle.

Finally, we also tested a *tol-1* mutant which is partially deficient in the only Toll-like receptor gene in *C. elegans*[165,64]. This class of receptors plays a major role in immunity of *Drosophila* and in *C. elegans* has been identified to be required in avoiding a product of pathogenic *Serratia* bacterium[165,163]. However, in case of PA14 avoidance *tol-1* is unnecessary (Fig. 4.14).

4.5 Conclusions

We showed that *odr-7* mutant animals are deficient in collective pathogen avoidance because they are incapable of detecting the alarm signal suggesting that AWA sensory neuron is used by the nematodes to detect the pheromone. Furthermore, *acox-1* animals were also unable to avoid bacteria faster in larger groups but in this case it was due to being unable to produce the signal. Because *acox-*

1 is involved in ascaroside biosynthesis this supports the hypothesis that the alarm signal is in fact a pheromone deposited into the lawn and detected directly by the AWA neuron. Alternatively, PA14 detect the product of *acox-1* dependent biosynthesis and in turn affect the behavior of the worms through AWA neuron.

Furthermore, we tested avoidance in mutant animals unable to produce four bioamines and their receptors that are used in *C. elegans* to regulate various behaviors. Although overall avoidance was affected in most mutants, no knockout was able to completely eliminate the collective avoidance suggesting that the regulation occurs through multiple pathways that could regulate different aspects of the avoidance behavior (e.g. attraction to food or repulsion from the pheromone).

Finally, we tested a number of innate immune mutants to see whether worms that are more susceptible to infection have a modified pathogen avoidance response. Each mutant was capable of collective avoidance (even though overall avoidance was affected in some mutants) suggesting that immune system may be regulated independently.

4.6 Methods

4.6.1 Strains and media

The following nematode strains were used in this study: N2 (referred to as 'wild type' in this and subsequent chapters), MT3762 (*osm-3(n1540) IV*), PR802 (*osm-3(p802) IV*), PR671 (*tax-2(p671) I*), CX10 (*osm-9(ky10) IV*), CX4544 (*ocr-2(ak47) IV*), JY243 (*ocr-2(yz5) IV*), PS6025 (*qrIs2 [sra-9::mCasp1]*), FK134 (*ttx-3(ks5) X*), CX4 (*odr-7(ky4) X*), CX3937 (*lim-4(ky403) X*), FK311 (*ceh-36(ks86) X*), DR476 (*daf-22(m130)II*), VS8(*dhs-28(hj8) X*), VS18 (*maoc-1(hj13) II*), VC1785 (*acox-1(ok2257) I*), CB1111 (*cat-1(e1111) X*), CB1112 (*cat-2(e1112) II*), MT14984 (*tph-1(n4622) II*), MT13113 (*tdc-1(n3419) II*), LX636 (*dop-1(vs101) X*), LX702 (*dop-2(vs105) V*), LX703 (*dop-3(vs106) X*), FG58 (*dop-4(tm1392) X*), CX13111 (*dop-5(ok568) V*), DA1814 (*ser-1(ok345) X*), OH313 (*ser-2(pk1357) X*), CX12800 (*ser-3(ad1774)*

I), AQ866 (*ser-4(ok512) III*), DA2100 (*ser-7(tm1325) X*), MT9668 (*mod-1(ok103) V*), INV63006 (*mod-5(n3314) I*) (MT9772 backcrossed 5x, a gift from Biron lab), RWK3 (*ser-1(ok345) X;ser-4(ok512) III;ser-5(tm2654) I;ser-7(tm1325) X;mod-1(ok103) V*)[88], CX13079 (*octr-1(ok371) X*), CX11839 (*tyra-3(ok325) X*), AU0028 (*fshr-1(ok778) V*)[162] (a gift from Ausubel lab), LT121 (*dbl-1(wk70) V*), (*zip-2(tm4067)*)[62] (a gift from Ausubel lab), VC1518 (*atf-7(gk715) III*), ZD101 (*tir-1(qd4) III*), KU25 (*pmk-1(km25) IV*), IG10 (*tol-1(nr2033) I*). All strains were obtained from the *Caenorhabditis* Genetics Center unless otherwise stated.

Worms were maintained at 25C (apart from *acox-1* which was kept at 15C) using the same protocol described in chapter 1. Same procedure as described in the previous chapter was used to prepare media.

4.6.2 Worm preparation

All worm strains were synchronized by hypochlorite treatment and first larval stage (L1) arrest for 12-16h[101]. Arrested L1s were transferred onto standard 6cm NGM plates (supplemented with 180 μg/ml streptomycin) seeded with OP50-1. In order for all strains to reach adulthood, *maoc-1*, *dhs-28* and *daf-22* worms were allowed to develop for 80h, 72h and 60h respectively at 25C, *acox-1* for 96h at 15C and the remaining strains for 48h at 25C.

4.6.3 Avoidance and conditioning assays

Avoidance and conditioning assays were performed in exactly the same way as described in chapter 1 with the exception that SK medium was supplemented with 50 μg/ml 5-fluorodeoxyuridine (FUdR) to prevent larval development. In the first stage of conditioning experiments 15-20 *odr-7*, *acox-1* or N2 worms were kept on the lawn for 18h using a metal ring.

5

Evolutionary conservation of collective avoidance

A progenitor of *C. elegans* N2 strain (whose descendants we used throughout this study) was collected in Bristol, UK in the early 1950s[154,238] and was maintained in laboratory conditions until the early 1970s when it was successfully frozen[23]. Throughout that time a selection pressure introduced a whole range of genetic modfications[232] into the organism and some SNPs have been shown to

change the behavior of the nematode[146] and in particular pathogen avoidance[35].

Collective avoidance is an adaptation that could potentially bring substantial survival benefits to *C. elegans* in the wild. Furthermore, it was recently shown that wild type isolates of other nematode species produce similar cocktail of ascarosides as N2[40]. Therefore, it is natural to ask whether these wild bacterivore species also exhibit collective pathogen avoidance or is it just an artifact of *C. elegans* domestication?

Furthermore, the fact that these different nematode species often can be found occupying the same habitats[76] and respond to similar pheromones[114] rises another interesting question - is there any interspecies communication occurring in the shared niches? More specifically, are other nematode species able to detect and react to *C. elegans* alarm pheromone and vice versa? This would open up a possibility of other species snooping on warning signals from their competitors or even tricking them into making decisions unfavorable for their survival using a common language of ascarosides. This would allow for the construction of a non-trivial interspecies competition model.

5.1 Background

Nematoda is one of the most diverse phylums of the animal kingdom. It is estimated that up to a million species of nematodes exist[128] even though only a few tens of thousands have been classified so far. They occupy a wide spectrum of habitats with varied natural histories ranging from deep oceans to arid deserts and from interstitial bacterivores to obligate parasites with multiple intermediate hosts[50].

Within the *Nematoda* an order *Rhabditida* evolved several major advances that set it apart from the rest of the nematodes and allowed it to dominate many new habitats[51]. A key adaptation within this group was the development of a chemically impermeable cuticle, which contributed to their success as parasites, colonizers and extremophiles. Another, was the development of an efficient

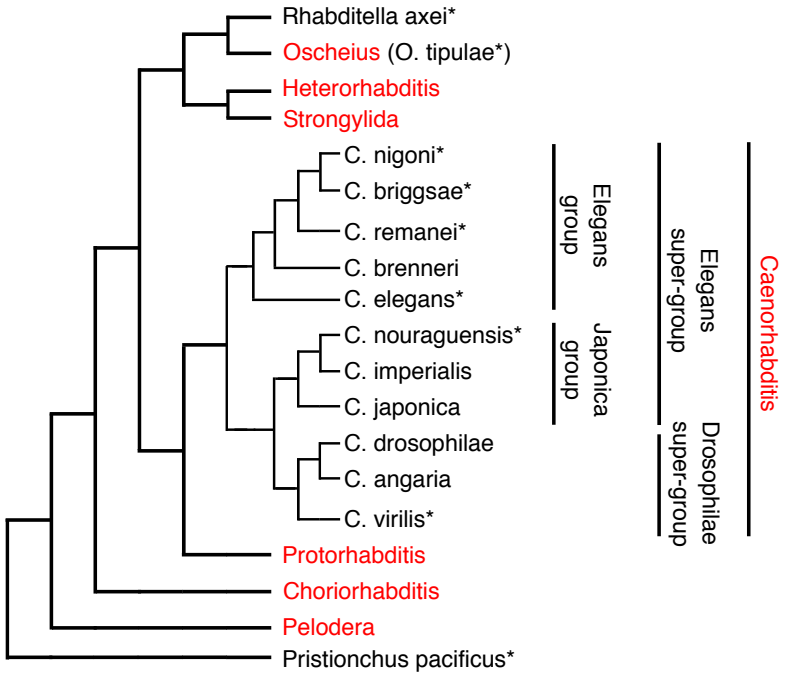

Figure 5.1: Phylogenetic tree of the family *Rhabditis* [120] and the genus Caenorhabditis [124]. Only selected clades (red) and species (black) are indicated. Species that were tested in this work are indicated with an asterix.

feeding pump - pharynx [140]. It is hypothesized that along with the reduction in size of the animal and shortening of overall generation time this organ allowed for a fast dispersion of these free living rapidly reproducing bacterivores [50].

In the order *Rhabditida* the suborder *Rhabditina* has developed a modified juvenile stage specifically adapted to long-term survival (in *Caenorhabditis* called dauer [103]). These non-feeding, highly modified and highly dispersive larvae are often capable of phoretically moving between distant environmental niches [121] on larger animals and also found many ways of invading internal organs of other organisms. Because this suborder contains *C. elegans* and at the same time does not lack in diversity, it has become a model for comparative genetics [203].

The detailed hierarchy was originally based on morphology and lifestyle and with the introduction of molecular phylogenetic techniques (based on sequencing of small subunit of rRNA [22] and

then other parts of rRNA and several highly functional proteins[51]) many taxa have been reclassified. Currently, as we understand it[124,123,122,120,22,21] *Rhabditida* contain not only the free living *Caenorhabditis* but also *Strongylida*, that harbor a number of highly infective human pathogens e.g. hookworm[20] and threadworm[54,229], *Heterorhabditis*, that are prolific arthropod parasites[161,42] and also a morphologically distinct diplogasterids that includes *Pristionchus pacificus* - a carnivorous nematode with a substantially modified pharynx, armed with teeth that is able to feed on other nematodes.

This diversity of *Rhabditida* is even more striking on the genetic level. Various studies have shown[120] that *Caenorhabditis* themselves are as varied as humans and fish at the genetic level. More broadly (Fig. 5.2), *C. elegans* is genetically as close to various members of *Oscheius* genus or *Rhabditella axei* as humans (*Homo sapiens*) are to sea urchins (*Strongylocentrotus purpuratus*).

Such a broad range of genetic variation encouraged work on closely and more distantly related nematodes for the purposes of comparative studies. *Caenorhabditis briggsae*[201,84], *Pristionchus pacificus*[182,198,100,144] and *Oscheius tipulae*[74,17] are examples of satellite models to *C. elegans* that are also easily cultivated in similar conditions[76]. This became a starting point for research on parasitic nematodes within *Rhabditida*[11] and beyond[110].

An excellent example of a comparative study that uses the power of these genetically diverse but ecologically similar biological models is research into production of ascarosides and their function between different species of *Rhabditida*. It turns out that different representatives of this order produce a diverse but overlapping set of ascarosides[40]. Moreover, some of the blends of different ascarosides have a similar effect on different species of animals[114] raising hope for future development of pheromone-based antihelminthic therapies.

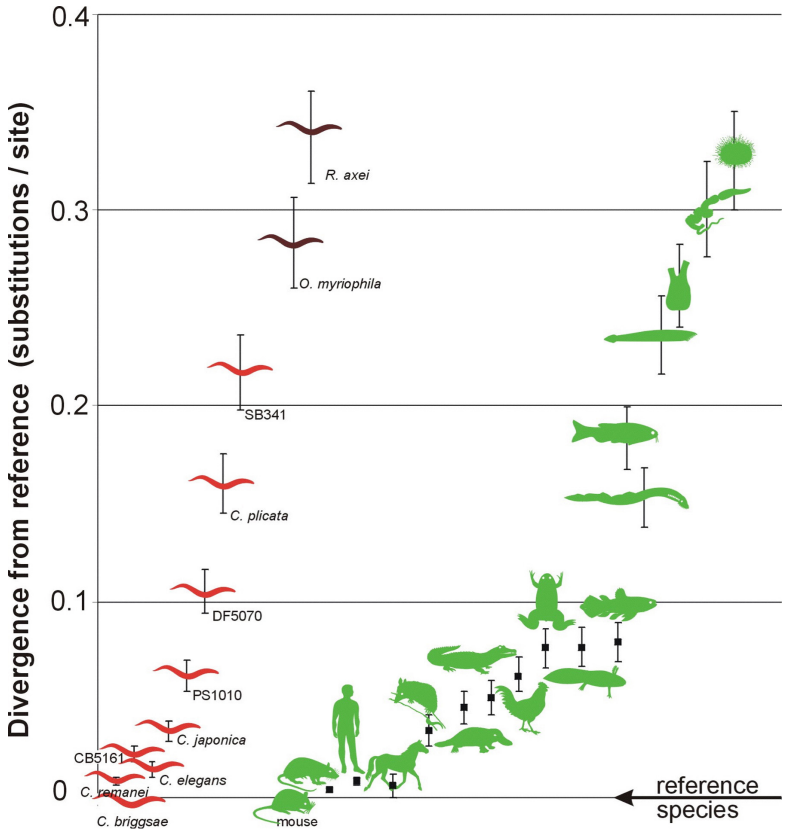

Figure 5.2: Comparison of genetic divergencies in nematodes (red) and deuterostomes (green) based on phylograms of SSU rRNA [120].

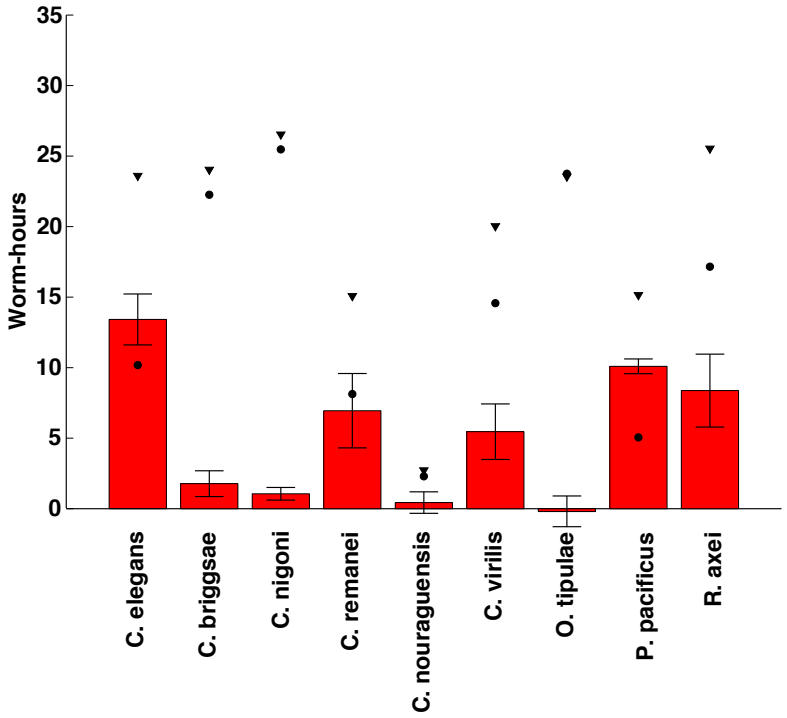

Figure 5.3: Collective avoidance for various *Rhabditida* species. The triangle (circle) shows the area under a multiple-worm (solitary) avoidance curve, the red bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

5.2 Conservation of collective avoidance in nematodes

To test whether collective avoidance is present in other nematodes we picked 8 other species of *Rhabditida* whose generation time was short, they could grow quickly on standard NGM plates with OP50-1 food, the different life stages were easily discernible, they were large enough to be imaged in our setup and at the same time reproduced the genetic diversity of this order of animals. The species we chose are marked with an asterisk in Fig. 5.1. From *Elegans* group of *Caenorhabditis* we chose *C. briggsae*, *C. nigoni* and *C. remanei*, from *Japonica* group we picked *C. nouraguensis* and from *Drosophilae* supergroup we selected *C. virilis*. Apart from *Caenorhabditis* we also used three species from other families: *O. tipulae*, *P. pacificus* and *R. axei*. These are all free living worms that feed on bacteria only (except *P. pacificus* which has teeth and can also feed on larvae of various nematodes). *C. briggsae*, *O. tipulae* and *P. pacificus* are primarily hermaphroditic (like *C. elegans*) whereas the rest are gonochoristic.

To synchronize each population before an avoidance experiment we picked 50-100 adult individuals (approximately equal number of male and female for gonochoristic species) to an NGM plate seeded with OP50-1 for 6-8h. After the eggs were laid we removed the adults and allowed the eggs to develop for 84h when each specie reached adulthood. Then we picked 1 or 16 adult gravid females (or hermaphrodites) to standard SK plates with PA14 (supplemented with FUdR) using the protocol from Chapter 4. We recorded avoidance for 36h 20min and the results are shown in Fig. 5.3.

Half of the tested species exhibit accelerated avoidance at higher population densities and the phenotype does not have a consistent evolutionary pattern as only two of them are within *Caenorhabditis* (*C. remanei* and *C. virilis*) and the other two are distant relatives of *C. elegans* and not closely related to each other (*P. pacificus* and *R. axei*). The remaining species show no significant dependence on the density with *C. nouraguensis* barely avoiding at all within the time of the experiment and the remaining three species rather quickly evacuating the bacterial lawn.

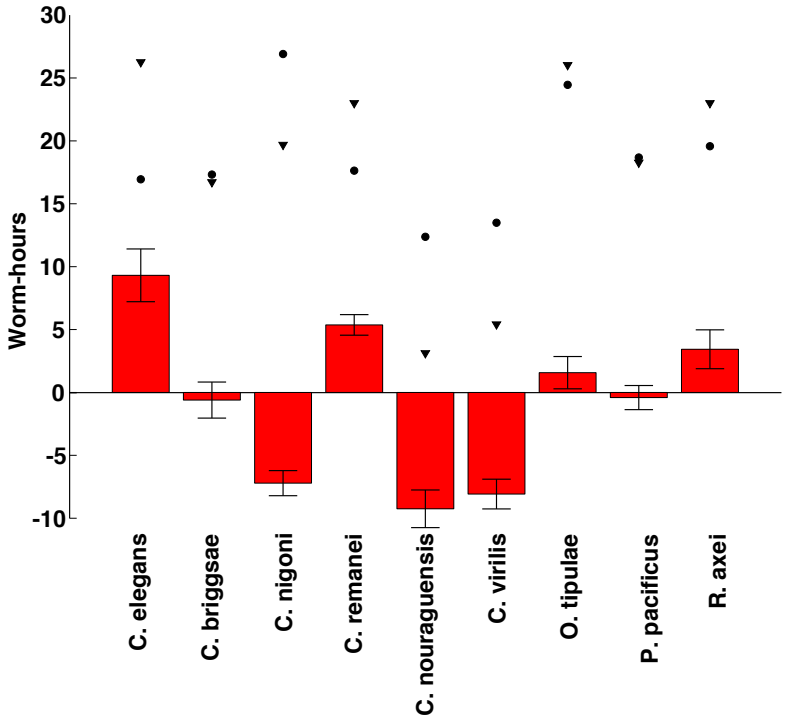

Figure 5.4: *C. elegans odr-7→Rhabditida* conditioning experiment. The triangle (circle) shows the area under an avoidance curve of 16 nematodes of a given species exposed to a mock-conditioned (conditioned with *C. elegans odr-7* worms) lawn, the red bar is the difference between them and the error is the sum of standard deviations in both conditions between multiple repeats.

5.3 Chemical communication between species of nematodes

To test whether the alarm signal left by *C. elegans* can be acted upon by the nematodes of another specie we performed a modified version of previously described conditioning experiments. First, we exposed PA14 bacteria on SK plates to *C. elegans odr-7* worms for 18h. These worms are capable of depositing the alarm signal (Fig. 4.6) but after 18h will still remain in the lawn (Fig. 4.5) which allowed us not to use the metal ring. After conditioning the *odr-7* worms were removed and 16 nematodes of another specie were placed in the middle of the lawn which were then imaged for avoidance. In parallel, another set of SK plates was prepared in exactly the same way except no worms were placed in the conditioning step - the lawn was simply allowed to age for 18h (mock-conditioning).

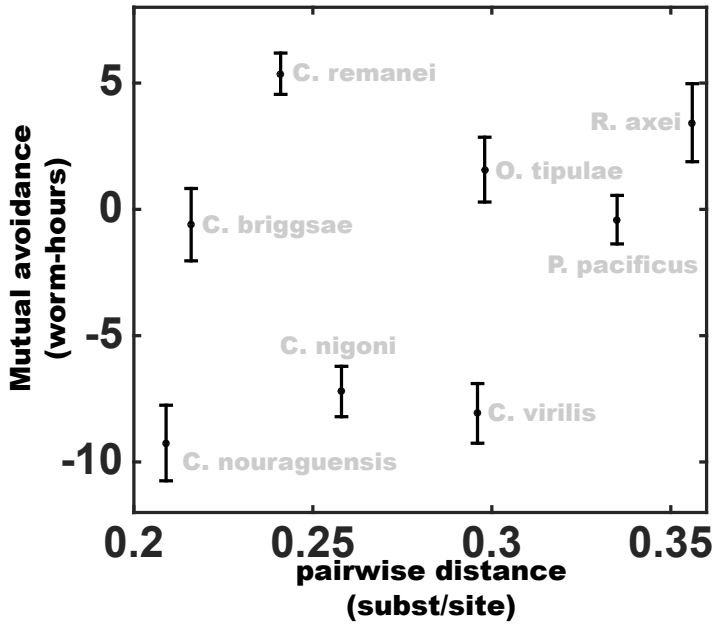

Figure 5.5: *C. elegans odr-7→Rhabditida* conditioning experiment result (vertical axis, the same as in Fig. 5.4) as a function of pairwise divergence computed using ClustalW [130] multiple alignment of RNA polymerase II mRNA sequences.

The results are presented in Fig. 5.4 and show the difference between mock-conditioning and conditioned lawns. This difference is positive if the signal left by the *odr-7* worms accelerates avoidance of naive nematodes (as is the case of *C. elegans*) and negative if the *C. elegans* alarm pheromone actually delays the avoidance.

Only two species (*C. remanei* and *R. axei*) heed *C. elegans* warning and leave the conditioned lawn faster than just the aged one. Three other ones (*C. briggsae*, *O. tipulae* and *P. pacificus*) are indifferent to the alarm pheromone. The remaining three (*C. nigoni*, *C. nouraguensis* and *C. virilis*) do the opposite to *C. elegans* and stay longer on the conditioned lawn compared to the aged one. This suggests that the *C. elegans* alarm pheromone has a varied effect on different *Rhabditida* species and this effect does not have a clear evolutionary pattern. The lack of conservation is also exemplified in Fig. 5.5 which shows the relation between avoidance of *C. elegans* alarm signal as a function of

evolutionary divergence between RNA polymerase II mRNA sequences.

To test whether *C. elegans* are able to react to the signal left by other species, we conditioned the PA14 bacteria with 15-20 worms of a given specie for 16 hours with a metal ring in place to force the worms to remain in the lawn. Then the ring and the worms were removed and replaced with 16 *C. elegans acox-1* mutants which are able to detect the pheromone but unable to produce it (later we repeated the experiments with N2 worms with the same effect). Unexpectedly, *C. elegans* did not leave the conditioned PA14 within the 20 hours of the experiment. This suggests that *C. elegans* is attracted by the signals left by all the tested species.

5.4 Conclusions

We have successfully shown that collective avoidance is not specific to the laboratory bred *C. elegans* nematode but in fact it occurs quite broadly among different *Rhabditida* species. Furthermore, the alarm signal left by *C. elegans* in the bacterial lawn is detected by most of the other species tested but some of them were attracted to it rather than repulsed. This suggests that although ascaroside communication exists in other nematode species, they interpret the same alarm pheromone in differently.

5.5 Methods

5.5.1 Strains and media

The following nematode strains were used in this study: *C. elegans* N2, CX4 (*odr-7(ky4) X*), VC1785 (*acox-1(ok2257) I*), *C. briggsae* AF16 (a gift from Needleman lab), *C. nigoni* JU1422 (formerly *C. sp. 9*), *C. remanei* PB4641 (a gift from Mango lab), *C. virilis* JU1968 (formerly *C. sp. 13*), *C. nouraguensis* JU2079 (formerly *C. sp. 17*), *O. tipulae* CEW1, *P.pacificus* PS312, *R. axei* DF5006. All strains were obtained from the *Caenorhabditis* Genetics Center unless otherwise stated.

Worms were maintained at 25C (except for *acox-1* which was kept at 15C) using the same protocol described in Chapter 4. Same procedure as described in the previous chapter was used to prepare media.

5.5.2 Worm preparation

odr-7 and *acox-1* worms were prepared using the same procedure as described in Chapter 4. The same hypochlorite treatment treatment for other species proved to be unreliable so we resorted to synchronization through egg-laying. We picked 50-100 adult individuals (approximately equal number of male and female for gonochoristic species) to an NGM plate seeded with OP50-1 for 6-8h. After the eggs were laid we removed the adults and allowed the eggs to develop for 84h at room temperature when each specie reached adulthood. Then we picked 1 or 16 adult gravid females or hermaphrodites (using a vulval plug as an indicator) to standard SK plates with PA14.

5.5.3 Avoidance assays

Avoidance assays were performed using the same protocol as in Chapter 4 except for experiments with *R. axei*. Because this specie was significantly larger than *C. elegans* and was feeding at faster rate, the PA14 inoculated on the plate was allowed to incubate for 8h (instead the usual 2h) on the plate before the start of the assay.

5.5.4 Conditioning assays

Avoidance assays were performed using a modified protocol from Chapter 4. For *odr-7* conditioning no metal ring was used (after 18h >90% of animals were still in the lawn and bacterial spreading was negligible). For conditioning with different *Rhabditida* species we used a different stainless steel washer (catalogue number 91944A106, obtained from McMaster-Carr) which was easier to handle.

5.5.5 Multiple alignment

Multiple alignment of RNA polymerase II mRNA sequences was done using ClustalW[130] program with default parameters. We used sequences with the following accession numbers: EU153426.1, EU153414.1, AY602183.1, JN636057.1, JN636126.1, M29235.1, XM_002634714.1, AH013929.2, JN636133.1.

6

Conclusion

In this work we developed a novel automatic imaging system capable of tracking the distribution of *C. elegans* nematodes and their bacterial food source on hard agar media. We used this setup to demonstrate that pathogen avoidance depends on the population density and that the signal is transmitted through the bacterial lawn. We have found that this collective avoidance is abolished in *odr-7* and *acox-1* mutants that were unable to detect or produce the signal respectively. This supports the hypothesis that *C. elegans* exposed to pathogenic bacteria secrete an alarm signal that is

detected through AWA neuron which drives the worms away from the danger.

The collective avoidance is not restricted to *C. elegans* but is exhibited by several other free-living nematode species within *Caenorhabditis* and also more broadly in *Rhabditida*. Although, the alarm signal deposited by *C. elegans* can be detected by many other species some are attracted to it rather than repulsed. This suggests that collective avoidance is used more generally by other *Rhabditida* species but the composition of the chemical signal is not conserved between them.

We have also developed a phenomenological model that is able to reproduce the quantitative features of collective avoidance behavior. The model assumes that the worms deposit the alarm pheromone into the bacterial lawn and the signal makes the nematodes more likely to leave the lawn in a concentration dependent way. Although the model is in a good agreement with the avoidance and conditioning experiments in the initial stages of those assays, it breaks down as the bacteria grow outside of the original lawn.

A natural extension of this work will be to identify the chemical nature of the putative alarm pheromone. This can only be achieved using mass spectrometry techniques. Once isolated, it will be possible to synthesize it and track down the exact set of neurons that the animal uses to detect it. It would also be interesting to see whether there exists a mix of ascarosides that could act as a universal alarm pheromone for parasitic nematodes. These could potentially be used in a similar way that the alarm pheromones are used to keep insects away from crops and could prove to be a formidable weapon agains plant, animal and human parasites.

The setup developed in this work could easily be used to track multiple populations of different nematodes in various behavioral assays. This could potentially open up the door to more studies of population-level dynamics on a quantitative level. As *C. elegans* are often found living in large populations in the wild this new quantitative approach coupled with a rigorous physical modeling could provide a valuable insight to biology of this fascinating model organism.

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