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C. elegans as a model for fear-like behaviors

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by

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The thesis of Amy Pribadi is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Abstract of the Dissertation

Caenorhabditis elegans as a model for fear-like behaviors

by

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Professor Sreekanth Chalasani, Chair

Learned fear has been studied in invertebrates including *Aplysia californica* and *Drosophila melanogaster*. Signaling using the biogenic amines dopamine and serotonin are conserved between invertebrates and vertebrates. While *Caenorhabditis elegans* is a model organism that has been extensively used to study mechanisms of learning and memory in aversive conditioning, these paradigms do not necessarily fall under learned fear. We have created a predator-prey paradigm using *Pristionchus* as a model predator and *C. elegans* as the model prey to study learned fear in *C. elegans*. We found that *C. elegans* exposed to

predator bites over at least six hours begin to leave a food patch and lay eggs away from it. After a twenty-hour exposure period, *C. elegans* retains this behavior for at least six hours after removal to an arena without any predators, indicating that this behavior can be learned. We found that dopamine signaling is required for the off-lawn leaving and egg-laying behavior. *C. elegans* deficient in dopamine synthesis are defective in performing the behavior and this defect can be rescued with exogenous dopamine treatment after predator exposure. Additionally, serotonin and mechanosensation appear to play a role in this behavior but their roles are not yet fully investigated.

Chapter 1:

Learned Fear in Invertebrates

1. Introduction

Animals are challenged with threats throughout their entire life. For example, animals may need to evaluate whether they are in a territory with predators or whether the food they are eating is toxic. Their nervous system senses external signals and integrates them with signals from within their body, uniquely equipping them with the ability to respond to threats with flexibility. Given a changing environment, a nervous system should also be able to identify new threats as they arise as well as forget them if they later prove to be harmless. These threats shape the evolution of behavioral programs, and underlying neural circuits and molecular mechanisms. While studies of the biological basis of fear are primarily conducted in vertebrates, invertebrate model organisms provide the potential for highly detailed, rapid studies not yet possible in higher organisms. In this review, we will discuss the study of learned fear in a laboratory with a focus on what has been accomplished in the invertebrates *Aplysia californica, Drosophila melanogaster,* and *Caenorhabditis elegans*.

The term “fear” has many definitions and different facets of it are emphasized depending on the field of study. In research using model organisms, most predominantly rodents, the learned association of an aversive, potentially harmful stimulus with a neutral stimulus is called “fear conditioning [1]. For simplicity of terminology, we will not distinguish between “fear” elicited by immediate threats and “anxiety” elicited by perceived threats [2],

[3], but rather group them together as aspects that both fall under the fear response. Following the common usage of the term “fear conditioning”, we will use “fear” throughout not to refer to the emotional state of the animal, which we cannot measure externally, but to the sum of behavioral and physiological responses that an animal undergoes when confronted with a threat.

The nervous system has evolved to adapt and respond to fear-inducing threats and instruct the animal to behave in a manner optimally suited to its current environment. The nervous system can change itself, sometimes structurally and/or chemically, to best fit the animal’s current challenges based on past experience. We will refer to the process of integrating past information to change future behavior as learning. We will refer to the process of integrating past information to change future behavior as learning. We will focus on behavior as a readout of learning, defined as a change to the internal representation of knowledge [4]. Learning can be either non-associative and associative [5]. The most rudimentary forms of learning are non-associative, meaning they involve changing behavior in response to only a single stimulus. Non-associative forms of learning include habituation, where the same stimulus is presented multiple times and the animal’s response to it diminishes over time, and sensitization, where the animal’s response to a stimulus increases upon subsequent presentation of the same stimuli. In contrast, associative learning involves learning a new relationship between two stimuli [5]. Associative learning typically allows the animal to assign new values to stimuli so that they can learn about new threats.

The systematic study of associative learning historically utilizes classical conditioning [6], [7], which involves using a stimulus that evokes a ‘hard-wired’ behavioral response, such as freezing in response to pain. This is called the unconditioned stimulus (US) because no prior conditioning is required to elicit the behavioral response. The experimenter then tests to

see if the animal can associate an unrelated stimulus with the US through temporal pairing of the two stimuli. The new stimulus is called the conditioned stimulus (CS) because the animal requires conditioning to respond to it. After successful training, when presented with the CS alone the animal will behave as if it is receiving the US. The interpretation of these results is that, through training, the animal learns that the CS predicts the US. Based on this interpretation, the probability of the CS predicting the US is directly correlated with learning ability [5]. This often means that multiple training sessions over longer periods of time will reinforce the association, resulting in more robust learning that is retained for longer. While classical conditioning often tests the extent to which animals are capable of pairing two unrelated stimuli, optimal learning requires us to consider the natural environment in which the animal has evolved to occupy. This means that different species should be more equipped to learn some tasks better than others depending on what cues are chosen. For example, rats are more likely to associate pain from shock with audiovisual cues, not gustatory cues. Conversely, they are more likely to associate nausea-inducing X-rays with taste, not audiovisual cues [8]. Honeybees can be trained with sugar water to discriminate between two colors, but odor overshadows color in learned discrimination tasks [5]. The sensory modality of the cues thus affects the ability for the organism to learn from them, as it depends on the organisms' innate abilities as well as its natural environment.

Assays probing fear in the laboratory must simplify the animal's natural environment to control interfering variables. Most learning studies involve breaking down the animal's experience into multiple epochs: training, testing, and recall. Acquisition of the new memory happens during the training period. The researchers then test the animals soon after training to determine whether the animal successfully acquired the memory. After this, researchers can test the animal's ability to recall the memory to determine retention over time. Additionally, certain experimental interventions such as presentation of the CS without the

US can extinguish learned memory quickly. However, memory extinction is beyond the scope of this review so it will not be considered here. Since it is difficult to know what is happening internally in the mind of the animal, measurable changes in behavior are often used to determine the success of learning.

Most naturalistic fear responses in rodents are studied in the context of predator-prey relationships. Live cats [9], cat odors [10], or even robotic predators [11] have been used. As mentioned above, the choice of fear-inducing stimulus is an important first step which requires consideration of the model organism's natural environment and the types of predators that they may encounter. Also, predator behavior has a profound influence on their prey. For example, ambush predators consume prey with different behavioral attributes than predators that actively hunt their prey [12] (the toadfish *Opasmus tau*, an ambush predator, preferentially consumed "shy" mud crabs *Panopeus herbstii* while the active hunter blue crabs *Callinectes sapidus* preferentially consumed "bold" mud crabs [13]). While prey behavior affects survival in context of differing predation strategies, prey must also balance their other needs: foraging and reproduction [11]. This cost-benefit calculation is likely a driving force in the evolution of prey behavior. Studying animals with simple neuroanatomy and robust behaviors allows these biological processes to be analyzed at the level of individual neurons, circuits and molecular pathways. While invertebrates typically are not thought of when considering fear, their usefulness in the laboratory has led to illuminating discoveries in the field. With this in mind, we will review some major findings in fear learning using invertebrate model organisms. We will first describe major findings obtained using studies in the sea slug *Aplysia californica,* the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans.* We will also summarize the similarities and differences between all three organisms and discuss how current models of learning in invertebrates fit into the broader study of fear and learned fear.

2. *Aplysia californica*

Early experiments in invertebrate learning were done in the sea slug *Aplysia californica*. This animal was selected due to its relatively few neurons (~20,000) and the fact that its neurons were easy to record from owing to their large size [14]. This model organism, specifically the gill-withdrawal circuit, helped to identify mechanisms of non-associative learning and memory. Repeated stimulation of the gill-withdrawal reflex diminishes its response (habituation) but a single shock to the head brings back the response (sensitization) without activating sensory neurons within the same circuit [15]. Further studies showed that this type of learning arises from a presynaptic change in calcium current rather than any structural change in the number of synapses within the circuit [16]. Through these experiments, it became clear that past experience of a neural circuit can alter the strength of signaling at the synapse through rapid intrinsic mechanisms without physical re-wiring of the existing circuit.

Aplysia also served as a useful model to determine the molecular basis of these intrinsic mechanisms. Studies found that serotonergic signaling increased cAMP levels [17], which activated PKA and altered presynaptic neurotransmitter release by increasing calcium influx [14]–[16]. These mechanisms can account for short-term behavioral effects without the need for protein synthesis. In contrast, memory that lasts beyond a day requires transcription and translation during the training period [18], [19]. Furthermore, cell culture studies showed that serotonin induces phosphorylation of the transcription factor CREB-1, which acts in the nucleus to induce transcription of selected genes that enforce long-term memory, providing a molecular link between short-term and long-term sensitization [14].

In addition, *Aplysia* can form associative memories through classical conditioning. Using the siphon- and gill-withdrawal reflex as a test circuit, a light touch to the siphon (CS)

produces a weak siphon withdrawal while a strong shock to the tail (US) produces a strong, longer-lasting withdrawal. The withdrawal response to light siphon touch is three times longer in animals trained with paired CS-US. Increasing the number of trials produces longer withdrawal responses, and the learned association is extinguished within ten trials of CS without US [20]. This training scheme is summarized in Figure 1. *Aplysia* can also form associative memories with odor. *Aplysia* are able to associate shrimp odor (CS) with head shock (US) proficiently after five trials [21]. Associative learning involves a larger circuit than the non-associative learning of the gill-withdrawal reflex because there are multiple inputs that need to be integrated. Studies using electrophysiological assays showed that the same activity-dependent presynaptic facilitation mechanisms identified in non-associative learning paradigms also play a role in associative learning [22]. Other researchers have demonstrated a role for postsynaptic mechanisms in associative learning in *Aplysia* for response specificity [23], [24].

The advantage of *Aplysia* is the ease of recording and direct electrical or chemical manipulations in their large neurons, but experiments on dissected preparations are not easily comparable with behavioral studies with intact, behaving animals [23]. Model organisms that are more tractable to genetic manipulation were instrumental in making this connection.

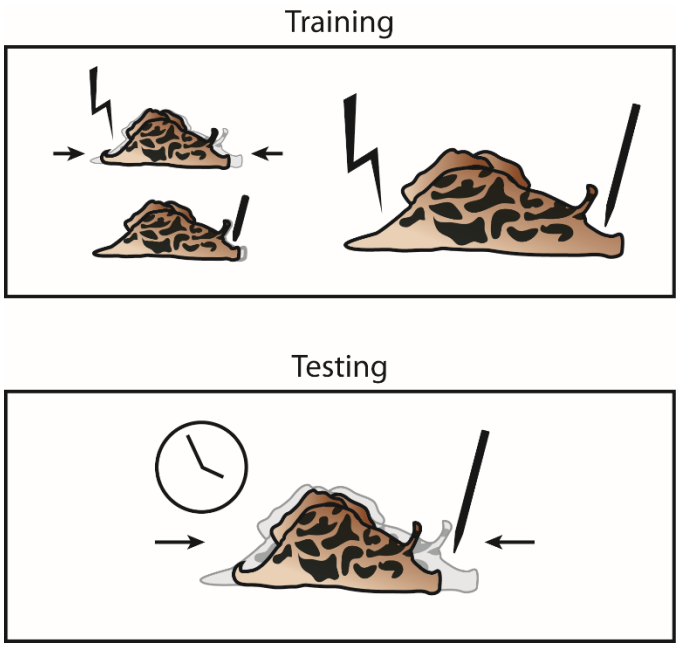

Figure 1.1 Classical conditioning in *Aplysia*. Training is performed by pairing electric shock with siphon touch. After training, siphon touch elicits an longer whole-body withdrawal response normally seen only with electric shock.

3. *Drosophila melanogaster*

The high fecundity and short reproductive cycle of the fruit fly *Drosophila melanogaster* make it an indispensable tool in identifying the genetic components of learning. Forward genetic screens identified many genes involved in different aspects of learning. Behavioral assays can also be conducted on populations of flies, allowing for more powerful studies. The development of tools for conditional gene expression unlocked the ability to explore the temporal and spatial specificity of learning and memory. The yeast-derived GAL4-UAS system and its variants enabled spatial control of transgene expression [25], [26]. Genetic screens have yielded libraries of mutants, including temperature sensitive mutants. An especially useful temperature mutation in *shibire*, a Dynamin ortholog, enabled circuit tracing with temporal control. Growing flies at a permissive temperature allows normal function of the gene. Shifting to the restrictive temperature blocks neurotransmission in neurons expressing *shibire*^{ts} [27]. The combination of selective gene expression and temperature sensitive mutations grants control of neural activity with both spatial and temporal specificity, and allowed researchers to study regions of the *Drosophila* brain and how they contribute to learning and memory.

Drosophila are capable of associative learning by pairing specific odors with shock. Using the odorants 3-octanol or 4-methylcyclohexanol as CS and electric shock as US, researchers found that flies specifically avoid entering tubes with the shock-associated odor. Memory of this training persists for at least an hour, and four spaced training events separated by two hours is sufficient to induce memory for at least a day [28]. This protocol was later modified to use a T-maze as a learning test after a training cycle of 60s odor exposure with or without inescapable shock followed by 30s of rest, and exposure to the second odor with or without shock. Learning in the T-maze is determined by comparing the

number of flies in the two collection tubes with either shock-paired odor or unpaired odor. This method of conditioning has a higher success rate, with maximal training achieved after a single training cycle [29]. This protocol, illustrated in Figure 2, is still widely used in memory studies [30].

A complete dissection of the circuits involved in fear conditioning to odorants requires identifying the pathways coding both odor and electric shock, as well as identifying the site of integration. The pathway for odor sensing can be summarized as follows: olfactory sensory neurons (OSNs) from the antennae and maxillary palps project to glomeruli within the antennal lobe, and projection neurons (PNs) from the antennal lobe form synapses with Kenyon cells in the mushroom body (MB) and terminate in the lateral horn. Within the antennal lobe, there are additional local excitatory and inhibitory interneurons that connect to the OSNs and span multiple glomeruli. The specifics of the olfactory circuit, particularly the anatomy of the MB, has been reviewed in-depth multiple times [30], [31]. Ablation of the MB results in memory defects but spares naïve avoidance of odors [32], so the MB appears to be an important site of integration downstream of odor sensation. The circuit for sensing electric shock is not known, but from *Aplysia* studies where shock induces serotonin release, aminergic signaling is likely involved. One study expressed the fluorescent calcium indicator cameleon in dopaminergic neurons and found that dopaminergic projections into the MB are activated by electric shock, and in trained flies these responses are prolonged for odor paired with shock [33]. Another study utilizing both *shibire*^{ts} and the GAL4/UAS system showed that blocking neurotransmission from dopaminergic neurons blocks aversive, but not appetitive, olfactory learning [34]. These studies suggest that dopamine, rather than serotonin, is the reinforcing signal for aversive learning.

Furthermore, unbiased genetic screens in *Drosophila* identified similar components to learning and memory as those in *Aplysia*. The cAMP/PKA pathway was identified in screens for learning and memory deficient flies [35]–[38]. In particular, the gene *rutabaga* encodes a type I Ca^{2+}/calmodulin-stimulated adenylyl cyclase that is theorized to be an important coincidence detector through detecting both Ca^{2+} increase and G protein signaling following monoamine binding [30], [39]. Expression of *rut* cDNA in the MBs can rescue the memory deficient loss of function mutant [40], [41].

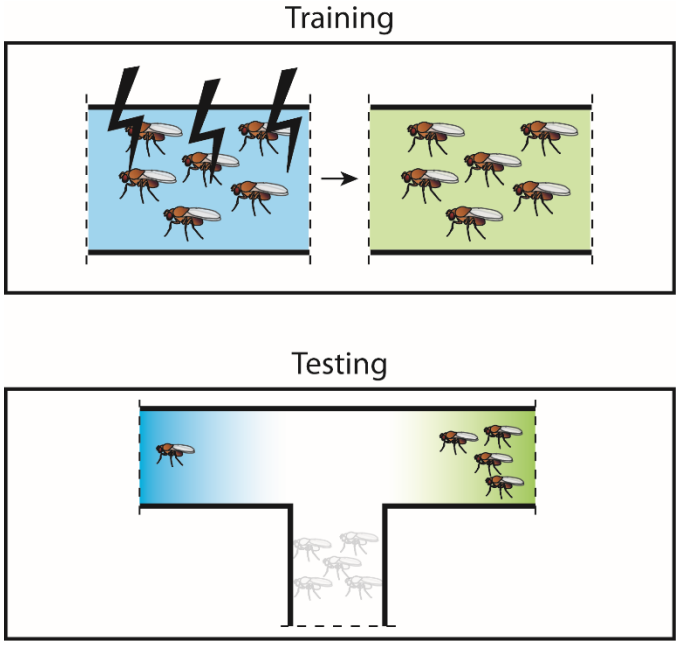

Figure 1.2 Olfactory fear conditioning in *Drosophila melanogaster*. Pairing electric shock with odor can alter olfactory preference.

4. Caenorhabditis elegans

An organism with even fewer neurons, the hermaphroditic nematode *Caenorhabditis elegans* is yet another useful model for studying neural mechanisms. *C. elegans* in nature are found in rotting organic material, such as fruits and stems, where they feed on the diverse microbes they encounter [42]. In the laboratory, *C. elegans* is grown in xenic culture with *E. coli* OP50 as food. Like with fruit flies, *C. elegans* behavioral studies are usually conducted with populations of whole, behaving animals rather than dissected preparations. With 302 neurons and a mapped connectome [43], *C. elegans* is an excellent model to study neural circuits on a single-cell basis. It also is a convenient genetic model due to its ability to package injected DNA into extrachromosomal arrays [44], [45], generating transgenic lines in a short period of time. The history of *C. elegans*' use as a genetic model also means that there exist libraries of mutants and lists of cell-specific promoters ready for use in experiments. While its neurons may be too small to easily patch, genetically encoded fluorescent calcium indicators can be easily imaged through its transparent body to study neural activity.

Conditioning with odor and food status

Studies in *Caenorhabditis elegans* have shown that the nematode can perform associative learning by pairing odors with food status. Odors such as 2-butanone and benzaldehyde, which are sensed by the AWC neurons, are normally attractive [46]. The behavioral test that measures attraction to the odor is a chemotaxis assay, an endpoint assay where the researcher places a population of animals between an odorant and its appropriate control. After around an hour of movement, the researcher determines the spread of animals relative to their position on the odor gradient [47]. If a population of *C. elegans* experiences one of these attractive odors in the absence of food, they will no longer

find that odor attractive [48], [49]. This experimental protocol is illustrated in Figure 3. Conversely, exposure to one of these odors in the presence of food can enhance attraction toward that odor [50], [51]. For this model of *C. elegans* associative learning, the training period with odor exposure in the absence or presence of food lasts for anywhere from thirty to ninety minutes. The extent of behavioral change increases with increased training time and plateaus at ninety minutes [48]. As in *Aplysia* and *Drosophila,* aminergic signaling has been shown to be involved during the training. Adding serotonin during odor conditioning mimics the effect of adding food, which normally blocks aversive learning. Mutants in *cat-4* that lack serotonergic and dopaminergic signaling have normal naïve approach and decreased attraction after training with starvation but adding food does not disrupt their training [50].

C. elegans show variable length of memory retention depending the duration of training. *C. elegans* trained for 60-80 minutes still exhibit trained behavior after 150 minutes of recovery, in contrast to animals trained for only thirty minutes [52]. This length of memory is dependent on cGMP-dependent protein kinase EGL-4. Mutants in this gene were found to be normal in naïve responses to AWC-sensed odors but defective in learning to avoid them when paired with starvation [53]. GFP-tagged EGL-4 enters the nucleus in AWC immediately after odor conditioning, suggesting that its nuclear translocation initiates learning [52]. Later research showed that EGL-4 phosphorylates proteins in the nucleus that promote the sustained change in behavior through RNA interference, providing a molecular link between early events during training and memory retention [54]. The memory of food status and odor associative training can be pushed even longer through spaced training, allowing the study of the mechanisms of long-term memory. Enhanced attraction to butanone due to association with food normally lasts around two hours, but the length of this memory can be increased by training in spaced blocks of food-butanone exposure separated by periods of

starvation. After seven such training blocks, memory as indicated by enhanced attraction remains for 16 hours. Cycloheximide and actinomycin D treatment blocked this long-term memory but not immediate memory, indicating that transcription and translation are required for the formation of long-term memory. Better long-term memory was also associated with increased levels of phosphorylated CREB [51]. While studies of adaptation to odor provided a foundational behavioral scheme that was easy to use and iterate through to discover genetic pathways, it is not the most naturalistic one. *C. elegans'* natural food does release some of the odors used in these odor conditioning experiments, such as 2-butanone [55] and isoamyl alcohol [56], but bacteria release odor blends rather than single odors [56].

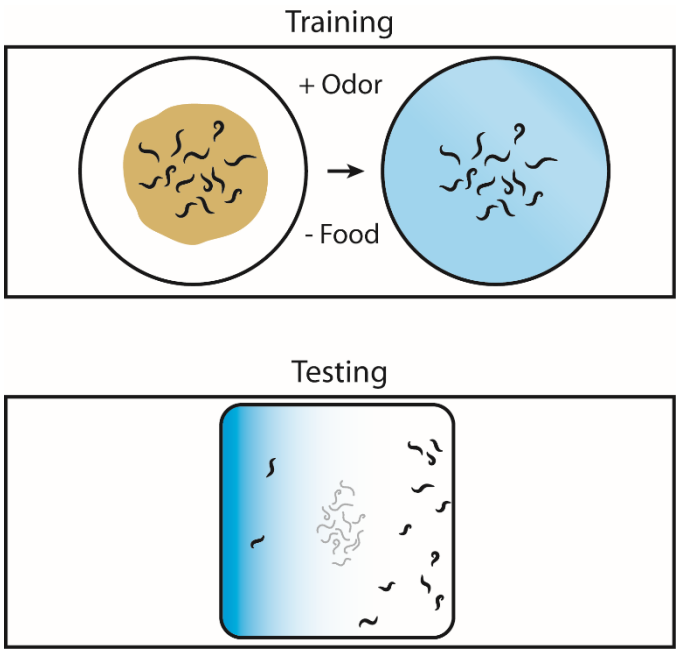

Figure 1.3 Aversive olfactory conditioning in *C. elegans*. When experiencing starvation and an odor, *C. elegans* can learn to associate odor with starvation. After this experience, they will move away from the odor.

Pathogen avoidance

Given the highly diverse microbial environment that *C. elegans* occupies [42], pathogenic bacteria is perhaps a more relevant threat than a single odor. To train *C. elegans* on pathogenic bacteria, they are exposed to a novel pathogen for four hours [57]. To test whether *C. elegans* learned to avoid this pathogen, they are confronted with a food choice assay where *E. coli* OP50 and the pathogenic bacteria are placed on opposite sides of an assay plate. A trained population of worms is placed in the middle of the plate and their locations relative to the bacterial patches after about an hour is recorded to determine their preference. This is illustrated in Figure 4.

The two most studied pathogens are *Pseudomonas aeruginosa* strain PA14 and various strains of *Serratia marcescens,* including Db10, Db11, and ATCC 13880. Attraction to *P. aeruginosa* PA14 is comparable to OP50 [57] in some setups and but it appears to be more attractive in others [58], [59]. *S. marcescens* is more attractive to naïve worms than *E. coli* OP50 [55], [57], [60], [61]. After training, *C. elegans* is repelled by these pathogens instead. It seems that toxicity is necessary for learned avoidance; most nonpathogenic strains of *P. aeruginosa* and *S. marcescens* do not induce learned avoidance [57], [62]. However, a mutant strain of *S. marcescens* derived from Db10 induces less learned avoidance despite being as virulent as its parent. This strain produces less of a compound called serrawetin W2 [60]. This suggests that toxicity induces learned avoidance, but the degree of that avoidance is not only dependent on virulence level but also on the chemicals released by the pathogenic bacteria.

What signals does the *C. elegans* use to determine whether their food is toxic? When considering pathogens, the innate immune system could signal if a bacteria strain is pathogenic. Indeed, a *tol-1* mutant with a defective TIR domain fails to avoid *S. marcescens*

Db11[63]. However, all the signals that that communicate to the nervous system that the food is toxic are not known. Serotonergic signaling is again important for acquiring this food-related memory; serotonin levels increase in ADF neurons after exposure to PA14, and this signal is necessary for aversive learning through the serotonin-gated chloride channel MOD-1 in downstream interneurons AIY and AIZ [57]. Neuropeptide release also plays an important part in modulating learning. Two neuropeptides play opposing roles to regulate RIA, another interneuron downstream of the identified learning circuit. INS-7 release from sensory neuron URX promotes learning by signaling to RIA and inhibiting DAF-2 activity. INS-6 release by ASI inhibits INS-7 release [59].

After the standard four-hour exposure to PA14, *C. elegans* continues to avoid PA14 odors for two hours [58]. Parents trained for twenty-four hours can transmit their learned avoidance of pathogenic *P. aeruginosa* to four generations of progeny via piRNAs [64]. However, the nature of a pathogen that is also food means that it can be evolutionarily advantageous to stop avoiding the pathogen if no better food is available. Upon exposure to PA14, increased expression of the neuropeptide INS-11 in the intestine inhibits strong learned behavior by inhibiting serotonin synthesis in ADF. Loss of function mutants in *ins-11* learn better but deplete their fat resources and lay fewer eggs than wild-type when PA14 is the only food available. Forgetting to avoid a pathogen that is also food allows adult animals to obtain enough energy to avoid starvation and lay viable eggs before succumbing to toxicity [62]. This apparent contradiction – encouraging forgetting but also very long memory – makes sense if considering that the strategy of forgetting toxicity in adulthood helps the eggs survive but transmitting memory to the next generation will encourage them to search for other food since they have time before reaching reproductive maturity. The study of pathogen avoidance is a fascinating example of considering *C. elegans*' natural environment to find which behavioral modifications are evolutionarily advantageous. Studying this

complex behavior in *C. elegans* has yielded many insights into the mechanisms of associative learning from the levels of sensory discrimination to behavioral execution. However, when considering a model of fear conditioning in *C. elegans*, it would be interesting to find a more acute threat that does not involve the complication of being both food and pathogen.

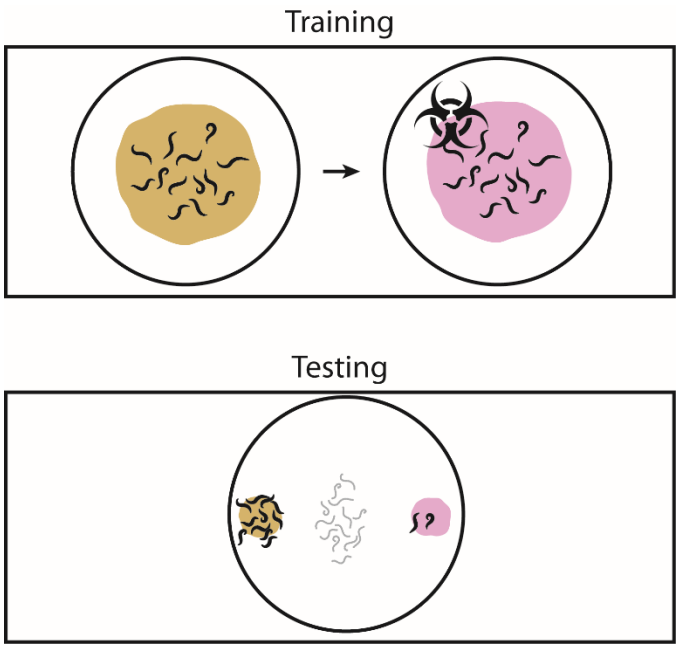

Figure 1.4 Learned pathogen avoidance in *C. elegans*. The yellow patch represents the *C. elegans'* normal food bacteria, OP50, while the pink patch represents pathogenic bacteria. *C. elegans* that have experienced pathogenic bacteria during a training session prefer the non-pathogenic bacteria, even if without training they may prefer the pathogenic bacteria.

Predation of *C. elegans*

Predation may be a way to study learned fear in *C. elegans*. Many types of fungi prey upon nematodes like *C. elegans*. Some fungi send out structures made of hyphae that can trap, paralyze, and digest the live nematodes. A 100-million-year-old fossilized sample of a nematode caught in a carnivorous fungi trap indicates that the relationship between nematodes and carnivorous fungi is ancient and widespread [65]. Through this coevolution fungi, developed advantageous mechanisms including luring their nematode prey with volatile cues that mimic food [66] and sensing pheromones released by *C. elegans* to increase trap formation [67]. *C. elegans* also has evolved mechanisms to escape death by fungi. Fungi like *Drechslerella doedycoides* send out constricting rings at the end of some hyphae that can catch *C. elegans* that crawl through them. The three cells that form the ring must inflate to successfully trap the nematode, but wild-type worms sense the ring and respond quickly enough to escape most of the time. The anterior touch response, which includes the worm both backing up and suppressing head movement, enables escape. Tyramine signaling is required to coordinate this behavior and mutants in a tyramine-gated chloride channel fail to escape carnivorous fungi as often as wild-type worms in a direct competition assay [68]. The study of *C. elegans'* behavioral responses to carnivorous fungi are an example of using the *C. elegans* to identify genes important to a population's survival in when faced with a natural predator. However, an encounter with predatory fungal traps either results in complete escape or death with little in-between. While it is possible that the *C. elegans* that successfully escape the traps might bear some memory of this event, this encounter is likely not particularly noxious.

Another predator of *C. elegans* is the nematode *Pristionchus pacificus. P. pacificus*, like *C. elegans*, is a bacterivorous hermaphrodite. Unlike *C. elegans*, *P. pacificus* is also a facultative predator of other nematodes. *P. pacificus,* as well as of other species in the

Pristionchus genus, have tooth-like structures that enable it to bite and consume their prey. *P. pacificus* show environmentally-influenced polyphenism in their tooth development. Depending on culture conditions, some develop into a narrow-mouthed stenostomatous (St) morph with a single tooth while others develop into a wide-mouthed eurystomatous (Eu) morph with both a dorsal and ventral tooth which facilitates predation of other nematodes [69]. We have previously shown that *C. elegans* can sense sulfolipids secreted by *P. pacificus* and avoids them [70]. *P. pacificus* could be interesting predator to evoke fear responses because, unlike carnivorous fungi, they can inflict sub-lethal damage on *C. elegans* [71]. *P. pacificus* is a relatively proficient killer of *C. elegans* larvae but they fail to kill *C. elegans* adults despite biting them at the same rate [72]. The bite of a predator like *P. pacificus* could be an unconditioned stimulus analogous to electrical shock, but unlike electrical shock, it is a threat that *C. elegans* may face in their natural environment. While *P. pacificus* is the most-studied nematode predator of *C. elegans*, it is important to consider whether *C. elegans* is likely to encounter *P. pacificus* in the wild at all. *C. elegans* is known to be found readily in decaying organic matter such as stems or fruits [42]. A study surveying the environments where *Caenorhabditis* are commonly found also discovered *P. pacificus* in samples of rotting stems and fruits [73]. The study of various *Pristionchus* species has primarily been used to study the evolution of features such as mouth form development and neural circuit pattern/function. We suggest that the *Pristionchus* – *Caenorhabditis* interaction might also be leveraged to study learned fear in nematodes.

5. Discussion

Research into learning and memory using these three invertebrate models has revealed shared patterns of memory acquisition and similar molecular players. Table 1 shows a comparison between experimental setups across the three invertebrates discussed above.

Table 1.1: Comparison of behavioral aspects of learning and memory research

Organism	Training	Behavioral test	Recall
Aplysia californica	Siphon touch (CS) and electric shock to tail (US)	Extended siphon withdrawal time	With spaced training over a day
Drosophila melanogaster	Odor (CS) and electric shock (US)	T-maze (avoidance of US-paired odor)	Over an hour; with spaced training over a day
Caenorhabditis elegans	Odor and food presence or absence	Chemotaxis assay (avoidance or enhancement of attraction to odor)	Around 2 hours; 16 hours with spaced training (enhancement)
	Pathogen avoidance	Food choice assay	Up to F_4 generation with 24 hours training

In both *Aplysia* and *Drosophila* studies the most common US is electric shock. While electric shock has the benefit of control of stimulus delivery, it is not one either animal likely encounters often in its natural habitat. The aversive nature of electric shock is different than the starvation/toxicity paradigms used in *C. elegans* studies. Electric shock delivers a single stimulus that is easily tunable by the researcher, but both starvation and toxicity are internal states that occur gradually over time. In *C. elegans* and *Drosophila*, odor is used as the CS. In pathogen avoidance, olfactory cues play a part due to the ability of the pathogen to attract or repulse animals at a distance but it is possible that gustatory or mechanosensory cues are also involved. In contrast, a light touch is used as the CS in the *Aplysia* experiments. The

behavioral tests for *C. elegans* and *Drosophila* are choice-based assays. They are scored based on the preference of a population of animals for a paired or unpaired stimulus. In contrast, the behavioral test in the *Aplysia* experiments was an exaggerated withdrawal reflex, not a choice. All three organisms have the capacity to retain long term memory lasting more than a day depending on the training regimen. Generally, training applied in spaced blocks induced long-term memory.

Shared mechanisms

A shared principle between in the study of memory is that fast changes can happen without requiring transcription or translation, but long-term memory does require transcription and translation. Generally, long-term memory is induced by early events during training that alter existing proteins (such as phosphorylation of CREB). These proteins then trigger an expression of a different set of genes that can reinforce the memory by restructuring the synapse through various mechanisms such as altering the type and number of receptors. A summary of some of the main mechanisms involved in learning and memory is illustrated in Figure 5.

Non-classical neurotransmitters such as biogenic amines and neuropeptides also play a role in acquiring and retaining memory. Serotonin codes food status in *C. elegans* and is released during gill-withdrawal stimulation in *Aplysia*. Dopamine reinforces punishment in *Drosophila.* Neuropeptides released by interneurons downstream of the sensory neurons can also modulate learning pathways by either improving or inhibiting memory formation. Research in invertebrates has provided a rich background for studying the mechanisms of learning. As reviewed above, many of the mechanisms and molecules used to store and enact learned behavior are conserved across species. Invertebrates have enabled these learning paradigms to be traced in detail from sensing to behavior, as shown in Figure 6.

Although the concept of fear as an internal emotional state may not apply to simpler organisms, this could also be argued for any non-human model organism. However, the ability to learn to predict and avoid threats should be useful for any model organism. Therefore, it is useful to consider fear from an exterior view as the behavioral response elicited by immediate threats. Invertebrates still have much they can teach us about learned fear, and utilizing the depth and breadth of learning and memory research in *C. elegans* while applying it to a fear conditioning context should yield fascinating results in the future.

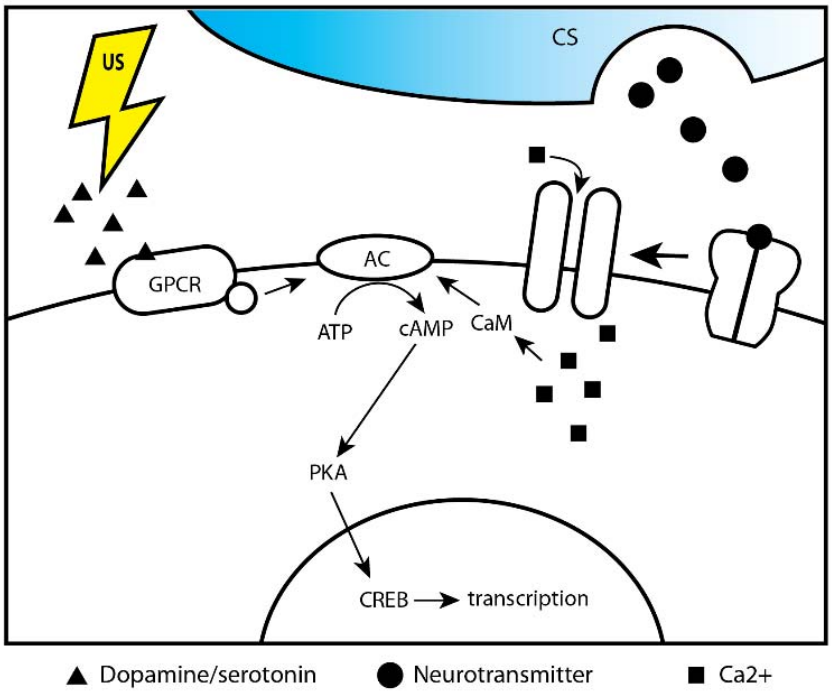

Figure 1.5 Conserved molecules in associative learning, as identified in invertebrate models. An unconditioned stimulus, like electric shock, causes release of dopamine/serotonin. The presence of a conditioned stimulus causes postsynaptic calcium levels to rise. Long-term behavioral changes can be effected through altered transcription via nuclear CREB.

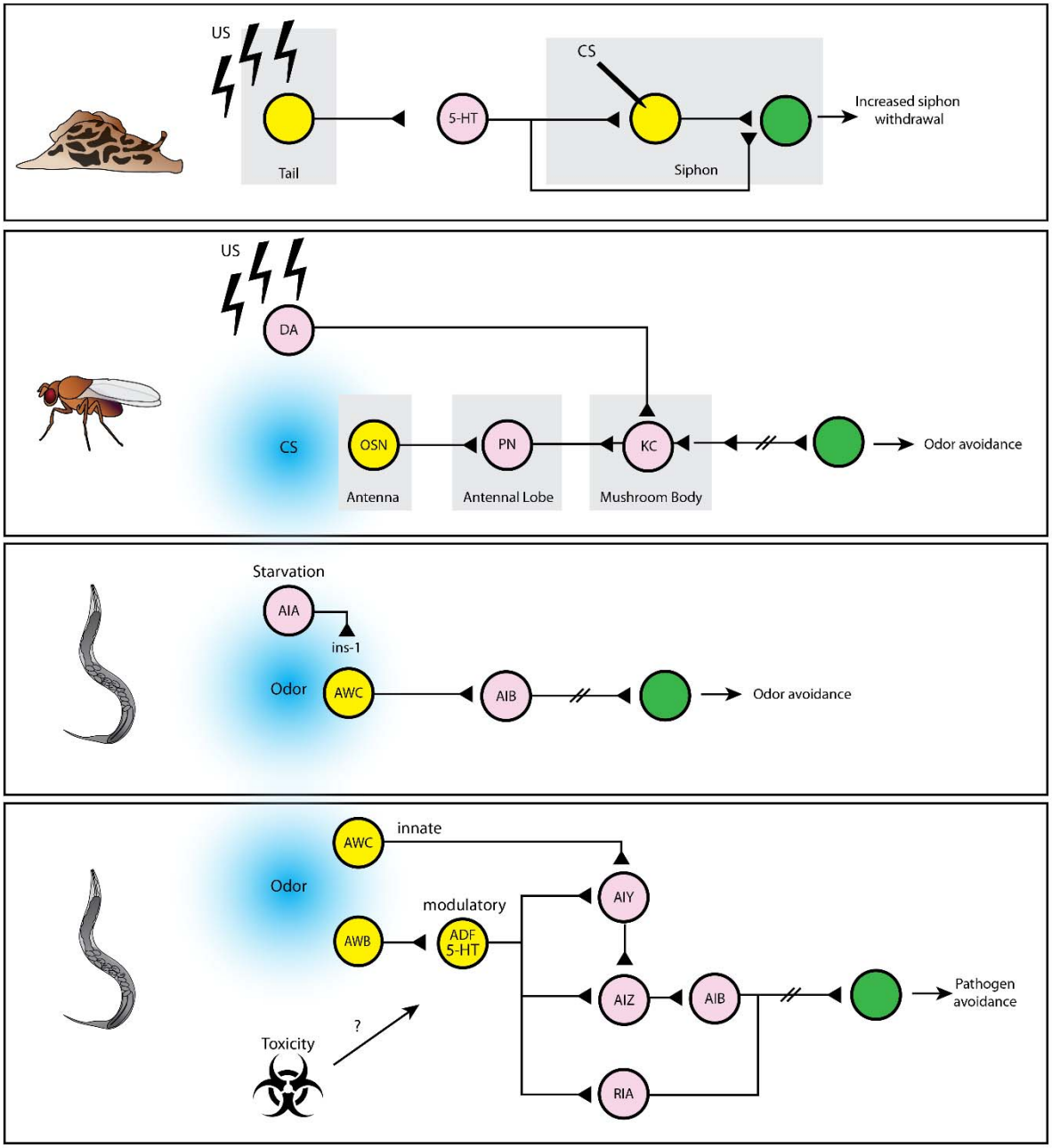

Figure 1.6 Circuit summaries for the training schemes discussed earlier. Circles represent neurons (yellow = sensory neuron, pink = interneuron, green = motor neuron) and the lines are their synaptic connections. Railroad lines indicate additional connections which are not illustrated. From top to bottom: first panel illustrates associative training protocol in *Aplysia.* US is electric shock to the tail and CS is touch to the siphon. Second panel: associative conditioning with odor (CS) and electric shock (US) in *Drosophila*. Third panel: starvation-induced olfactory avoidance in *C. elegans*. Odors sensed by AWC during starvation become aversive after training. Fourth panel: pathogen avoidance training in *C. elegans*. After exposure to pathogenic bacteria, *C. elegans* avoids odors released by the pathogen using both an innate pathway and a modulatory pathway.

6. References

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Chapter 2:

A dopamine-driven fear learning paradigm

1. Introduction

A key aspect of the nervous system is the ability to predict positive or negative stimuli, and then to act on this information appropriately. Learning involves integrating past experience with current information to predict future stimuli. Fear learning, which results from conditioning with an aversive, harmful stimulus [1], is an integral part of deciding upon behavioral strategies to maximize the use of the environment while minimizing risk.

Caenorhabditis elegans is a nematode that lives in rotting vegetation, eating the bacteria there [2]. With 302 neurons and a mapped connectome [3], it is an excellent model to study neuroscience down to the single-cell level. The majority of aversive learning research conducted in *C. elegans* focus on starvation or pathogenicity as the threats. For example, *C. elegans* can learn to avoid a specific odor if it experiences starvation and the odor at the same time [4], [5]. Also, *C. elegans* can learn to avoid pathogenic strains of *Pseudomonas aeruginosa* and *Serratia marcescens* once it experiences toxicity [6], [7]. However, viewing these studies in a fear learning context is complicated by the fact that the negative stimuli – starvation and toxicity – are complex internal processes rather than direct outward threats. We wondered whether we could use a predator instead to create a model for learned fear in *C. elegans*.

Behaviors evoked by predators vary by species, depending on predator hunting style [8] as well as sensory modality of the cues in relation to the prey's abilities and natural environment [9]. Prey also must evaluate the risk-benefit reward of engaging in antipredator behaviors, which often involve forgoing food or mating. For example, rats confronted with cat odor will spend more time in shelter and less time exploring [10]–[12]. Considering that most research in prey-predator relationships involve organisms that rely on sight, the behaviors that will be seen in olfactory/mechanosensory-dependent organisms like *C. elegans* should be viewed separately than the behaviors exhibited by other organisms. With this in mind, we conducted this study to look for *C. elegans*-specific behaviors that may not necessarily line up with traditional fear-associated behaviors like freezing.

Pristionchus are a facultative predator found in necromenic association with beetles [13] as well as in rotting vegetation where *Caenorhabditis* are also found [14]. They are able to subsist on bacteria alone [15] but can also kill and consume other nematodes including *C. elegans* [16]. Members of the *Pristionchus* genus exhibit mouth polyphenism. *Pristionchus pacificus* exhibit either a two-toothed Eurystomatous (Eu) mouthform or a single-toothed Stenostomatous (St) mouthform [17]. The Eu mouthform enables biting, and potentially killing, of nematode prey like *C. elegans* [18], [19]. Environmental conditions such as food availability and media type can alter the Eu/St ratio. Under standard culture conditions on solid NGM with *E. coli* OP50 as food, the majority of *P. pacificus* exhibit the Eu mouthform [20].

While *P. pacificus* is a relatively well-studied species within *Pristionchus,* it is uncertain whether *C. elegans* actually interacts with *P. pacificus* in nature. In contrast, the gonochoristic species *Pristionchus uniformis* has been found in the same sample with wild *C. elegans* isolates [14] so it is likely that the two species may encounter each other in nature.

P. uniformis was first characterized as a St-only species [21] but was recently recharacterized as possessing either a bacterivorous St or predatory Eu mouthform [22]. We found that in standard growth conditions most *P. uniformis* strain JU1051 are Eu, and the ratio is similar to that found in *P. pacificus* (Figure 2.1).

Fear conditioning experiments in *Drosophila melanogaster* pairing electric shock and odor show that dopaminergic signaling in the mushroom body appears to be the reinforcement signal for fear learning [23]. Traditionally, dopamine has been viewed as a molecule representing reward. With further studies, the role of dopamine has become more expanded and more nuanced. Dopamine release can be tonic or phasic and the binding properties of various dopamine receptors leads to different response profiles to tonic or phasic dopamine release [24]. Phasic dopamine release is associated with reward prediction but is also associated with aversive stimuli like pain. To reconcile these roles of dopamine, current models propose that phasic dopamine codes salience regardless of valence – the spike in dopamine alerts the organism to something important and unexpected so that the organism may recalibrate its expectations [25].

In *C. elegans*, dopamine modulates behavior based on environmental conditions and internal state. One of the first identified roles for dopamine in *C. elegans* is slowing down when entering food. This behavior is called basal slowing [26]. Dopamine also influences how *C. elegans* searches for food after its removal. When *C. elegans* are removed from food, they conduct an area restricted search by increasing turns for a short period of time. This helps them to find the food again. If the *C. elegans* fails to find food during this area restricted search, it switches to a global search strategy and instead suppresses turns. This strategy lets them search a larger area. Animals with dopaminergic neurons killed do not show this

transition to global search behavior, and treatment with exogenous dopamine rescues this [27].

Dopamine has also been shown to modulate learned responses to aversive stimuli. For example, dopamine release sensitizes aversive responses to multiple soluble repellent stimuli including copper and glycerol [28]. Dopamine also delays habituation to repeated taps [29], [30] and state-dependent olfactory adaptation during ethanol exposure [31] . Dopamine is generally thought of as the food sensor, so in this way it which provides context for appropriate behavior – it may be more beneficial to alter learning rate when on food versus when off food.

We have previously shown that *C. elegans* can detect and avoid sulfolipids released by *P. pacificus.* They also display a transient decrease in egg-laying after a thirty-minute exposure to sulfolipids that is blocked by the serotonin reuptake inhibitor sertraline [32]. However, while avoidance could be elicited by low concentrations of sulfolipids, the egg-laying effect required more concentrated sulfolipids, which probably are not encountered often in nature. We have also shown that *P. pacificus* is able to induce a change in egg distribution relative to food, encouraging more off-lawn egg-laying than normal [33]. We wondered this more naturalistic setup of prey encountering predators could elicit learned responses, and if biogenic amine signaling like dopamine signaling could modulate it.

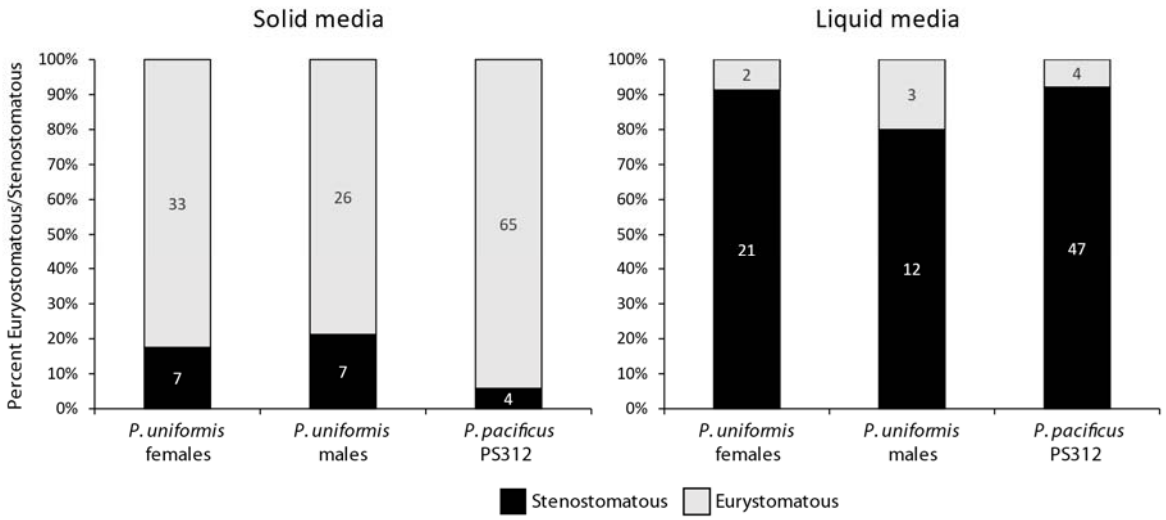


Figure 2.1 *P. uniformis* males and females are primarily in the predatory Eurystomatous form when grown on standard solid media. Growth in liquid media shifts this ratio to mostly bacteriovorous Stenostomatous, as it does in *P. pacificus*.

2. Results

2.1. Spatial egg distribution

Using a modified version of the protocol in [33], I exposed *C. elegans* to predators by placing three predators and three *C. elegans* on an assay plate containing a small, dense bacterial lawn (Figure 2.2). Control plates had six *C. elegans* to maintain a consistent number of worms between plates with predator and without. A ¼" filter paper corral was placed around the lawn to constrain the animals' movements and force them to interact more often. There was a small area of bare agar between the bacterial lawn and the corral. The area inside the corral was imaged at the relevant time points to observe the spatial distribution of eggs. Since *Pristionchus* also lay eggs, we used a *C. elegans* strain with an integrated GFP marker that expresses in eggs (*Pelt-2*::GFP).

To observe to what extent predator type affects *C. elegans* prey behavior, I chose several different types of predators*: P. pacificus* strains PS312 and RS5194, a St-only *P. pacificus* mutant TU445 *eud-1(tu445)* [34], and an isolate of *P. uniformis*, JU1051. *P. pacificus* strain RS5194 is more aggressive than PS312 as characterized by an increased probability of bite per encounter [35] so both strains were included in this analysis. The St-only mutant was included to demonstrate whether mere presence of a predator could alter *C. elegans* behavior, or if the predator must be able to bite. Finally, I wondered whether *P. uniformis*, which may interact with C*. elegans* in nature, could induce different behavioral changes. *P. uniformis* males and females were considered separately, while only the more common hermaphrodites were selected as *P. pacificus* predators.

I first tested if short-term exposure could increase off-lawn egg-laying. I found that, after six hours of exposure, only modest changes in egg distribution could be seen in *C.*

elegans paired with RS5194, the more aggressive [35] *P. pacificus* strain (Figure 2.3). To prevent eggs hatching into L1s, which secrete pheromones that promote lawn-leaving [36], the assay only ran for six hours.

To increase predator exposure time, I conducted the assay with L4 *C. elegans* and J4 *Pristionchus* instead of adults and stopped the assay after 20 hours of exposure. The juveniles developed into adulthood over the course of the assay, and *C. elegans* laid eggs only in the latter portion of the assay. Using the 20-hour assay, we found that all *Pristionchus* except for the St *eud-1* mutant were able to increase the median distance of eggs laid from the lawn edge (Figure 2.4 A). These experiments showed that *C. elegans* changes their egg distribution relative to the lawn when paired with a predator for more than six hours. These behavioral changes require a predator capable of biting.

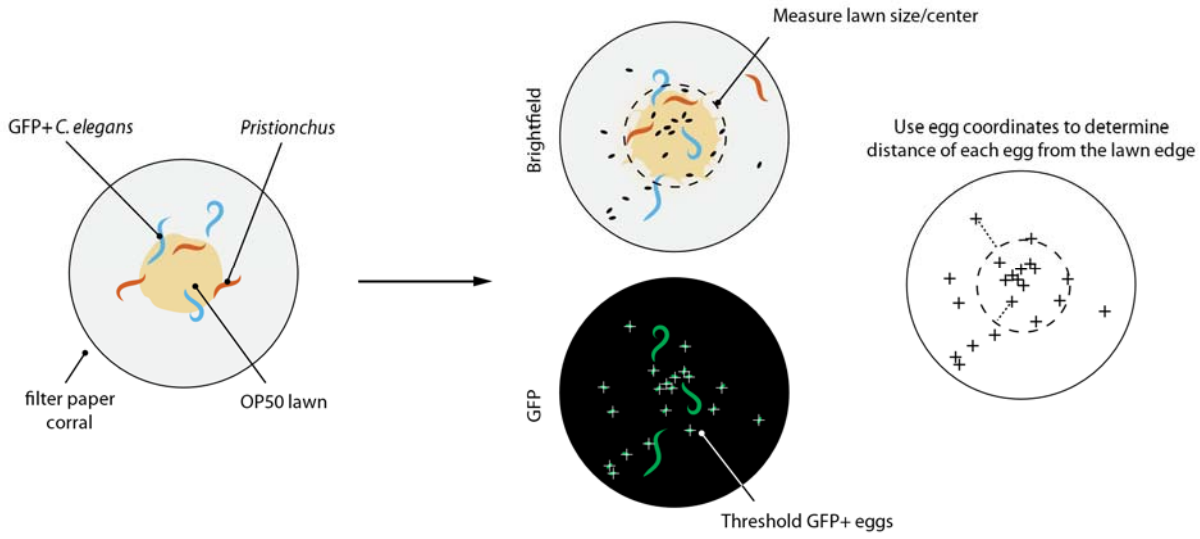


Figure 2.2 Outline of the egg distribution assay. Three *C. elegans* expressing *Pelt-2*::GFP are paired with three *Pristionchus* in a ¼" arena with a small OP50 lawn in the center. The animals are allowed to interact and *C. elegans* eggs are identified through GFP expression. Egg locations are then manually marked and distances to the lawn edge are calculated.

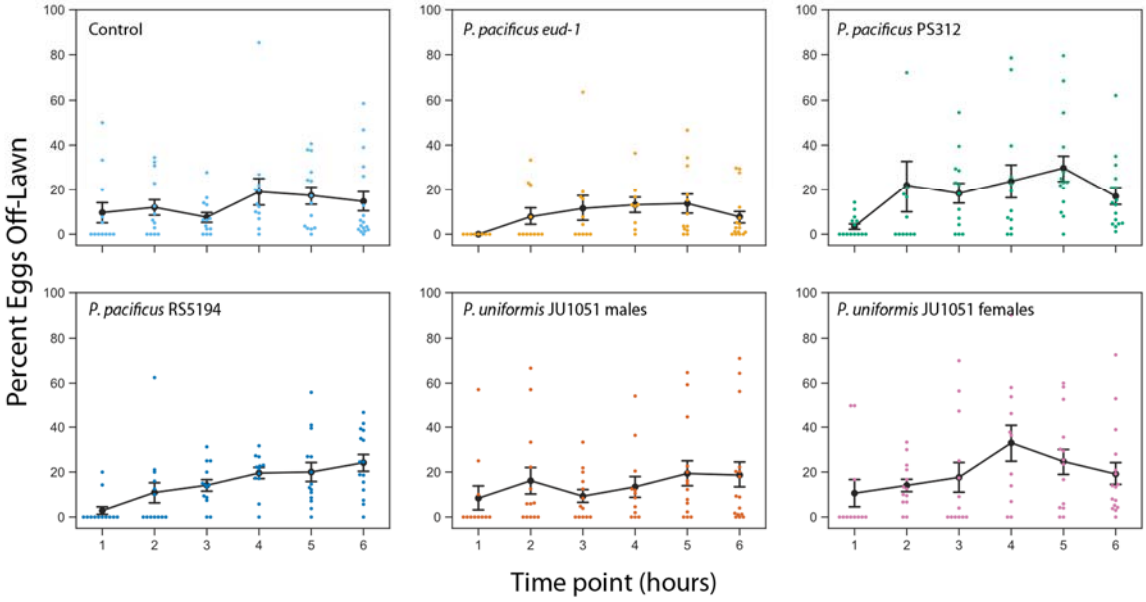


Figure 2.3 Short-term egg distribution assay results for each hourly time point up to six hours. Each data point represents a single assay. Error bars represent SEM.

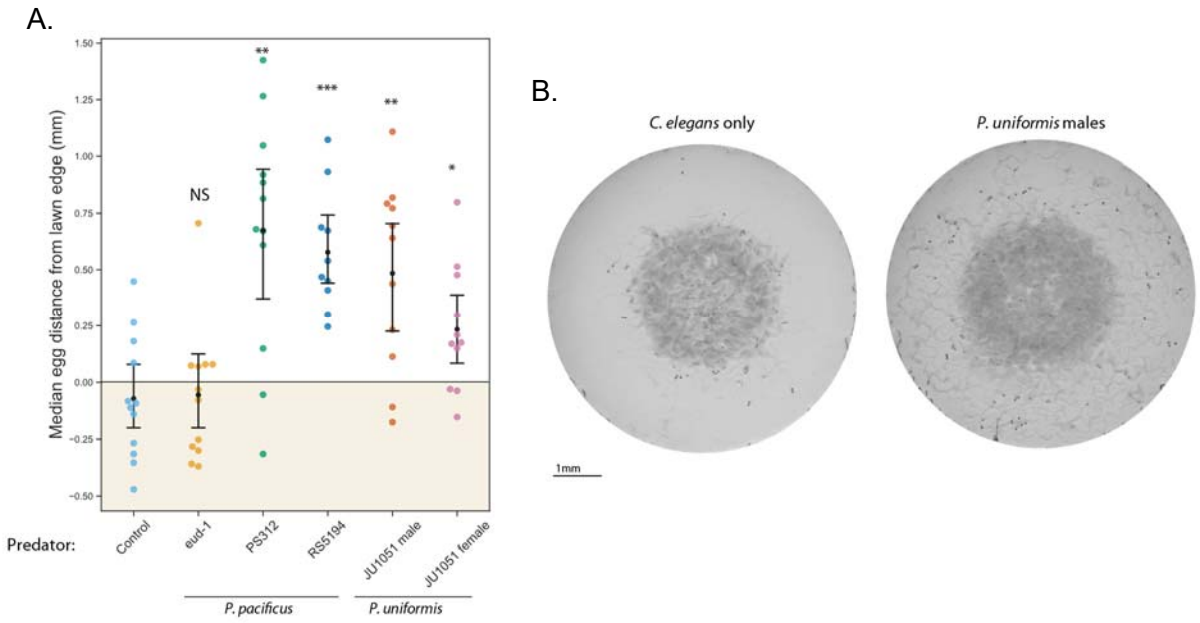


Figure 2.4 A. Overnight (20-hour) egg distribution assay results with various predators. Each data point represents the median distance of the eggs to the lawn edge for one assay plate. Error bars represent 95% confidence interval. Pairwise comparisons are Welch's t-tests comparing control (*C. elegans* only) to each predator type. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$. **B.** Representative images of assay plates after the 20-hour assay, with C. elegans-only control on the left and *C. elegans* paired only with *P. uniformis* males on the right.

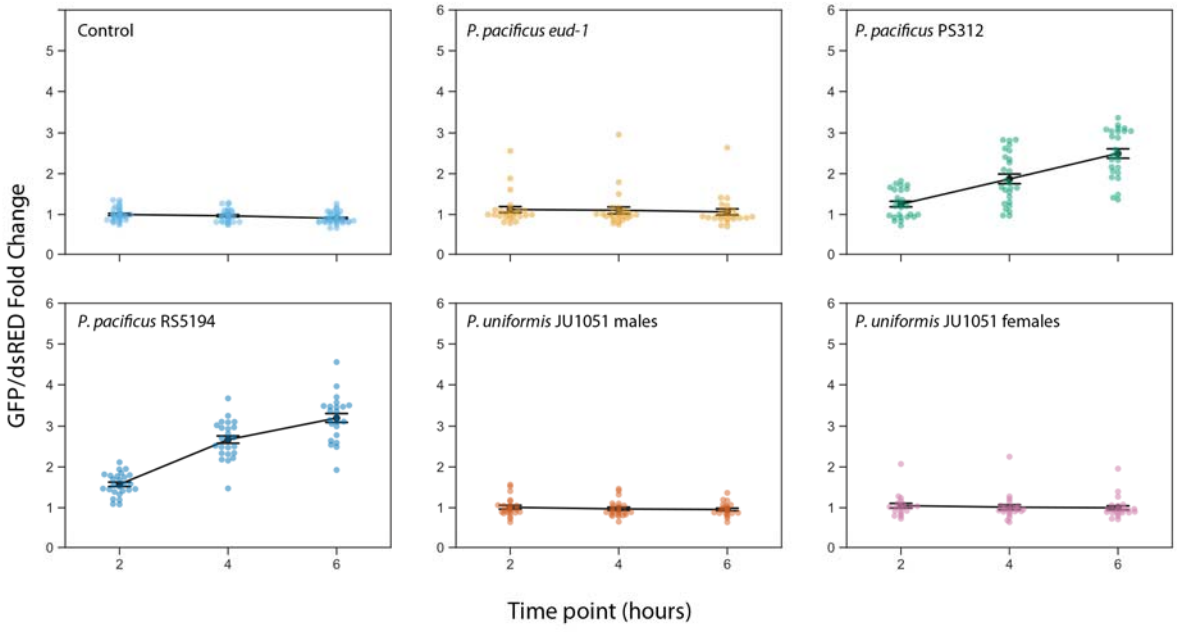
Injury is not required

It is possible that physical injury to the body of the *C. elegans* could affect egg distribution behavior. To test whether injury level correlates with this behavior, I used a *C. elegans* reporter strain expressing GFP under control of the *nlp-29* promoter. *nlp-29* expression is upregulated following injury due to fungal infection, laser, or piercing with a microinjection needle [37], [38]. I paired each predator type with the *Pnlp-29*::GFP *C. elegans* and measured GFP expression normalized to the dsRED coinjection marker. I found that in the shorter six-hour assay, both PS312 and RS5194 strains of *P. pacificus* were able to induce GFP expression by four hours but the St- only *P. pacificus* could not. The *P. uniformis* males or females also did not induce any change in GFP expression (Figure 2.5 A). Even during the overnight assay, the *P. uniformis* strains as well as the St- only *P. pacificus* failed to induce any GFP expression (Figure 2.5 B). The *Pnlp-29*::GFP strain also probably had some underlying behavioral defect which made it worse at escaping bites, or perhaps some other defect that made it more susceptible to bites, as when paired with the more aggressive RS5194 strain overnight, no survivors were found to measure. Taken together with the behavioral results, it appears that the sensation of bites without any cuticle penetration is enough to shift egg-laying distribution, and it takes at least six hours for behavioral change to occur.

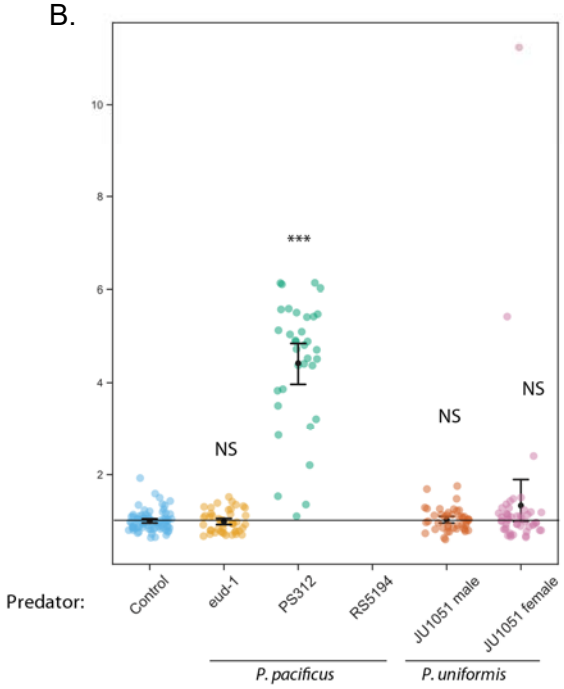
I decided to use *P. uniformis* males as the main predator for the rest of my studies (Figure 2.4 B). *P. uniformis* males do not lay any eggs, no longer requiring the use of the *Pelt*-2::GFP marker. *P. uniformis* males also do not induce injury, so behavioral changes can be interpreted without having to account for injury itself causing the behavioral change.

Figure 2.5: Different predators have different capabilities to cause injury. Injury is reported using *C. elegans* expressing *Pnlp-29*::GFP. GFP measurements are normalized to *Pcol-12*::dsRED co-injection marker. Fold-change of fluorescence is shown relative to the mean of the *C. elegans*-only control. **A.** During the six-hour assay, PS312 and RS5194 can incur injury. Error bars represent SEM. **B.** After the 20-hour assay, only PS312 induces injury. RS5194-exposed animals are missing because they die after 20 hours of exposure. Error bars represent SEM. Pairwise comparisons were performed with a Welch's t-test between each predator type compared to control no-predator condition. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

A.


Control
P. pacificus eud-1
P. pacificus PS312
P. pacificus RS5194
P. uniformis JU1051 males
P. uniformis JU1051 females
GFP/dsRED Fold Change
Time point (hours)

B.


***
NS
NS
NS
Predator:
Control
eud-1
PS312
RS5194
JU1051 male
JU1051 female
P. pacificus
P. uniformis

Predator Ratio

I next observed how the ratio of predators influenced this egg distribution behavior. Maintaining the total number of worms on the assay plate at six, I varied the ratio of *C. elegans* to *P. uniformis* males. I found that adding a single predator was able to shift the egg distribution behavior and adding two or more resulted in off-lawn egg laying that was similar across different ratios (Figure 2.6 A). It is possible that the behavior could have increased with increased predators in a larger arena, which would have allowed a greater increase in the distance possible. I also considered that the egg distribution may be influenced by the total number of eggs laid during the assay time. However, the presence of predators, even many predators, did not affect the total number of eggs laid (Figure 2.6 B). I decided to use three predators and three *C. elegans* in further studies to ensure a strong effect of predator on egg distribution behavior while also keeping a relatively high number of eggs on the assay plate to measure.

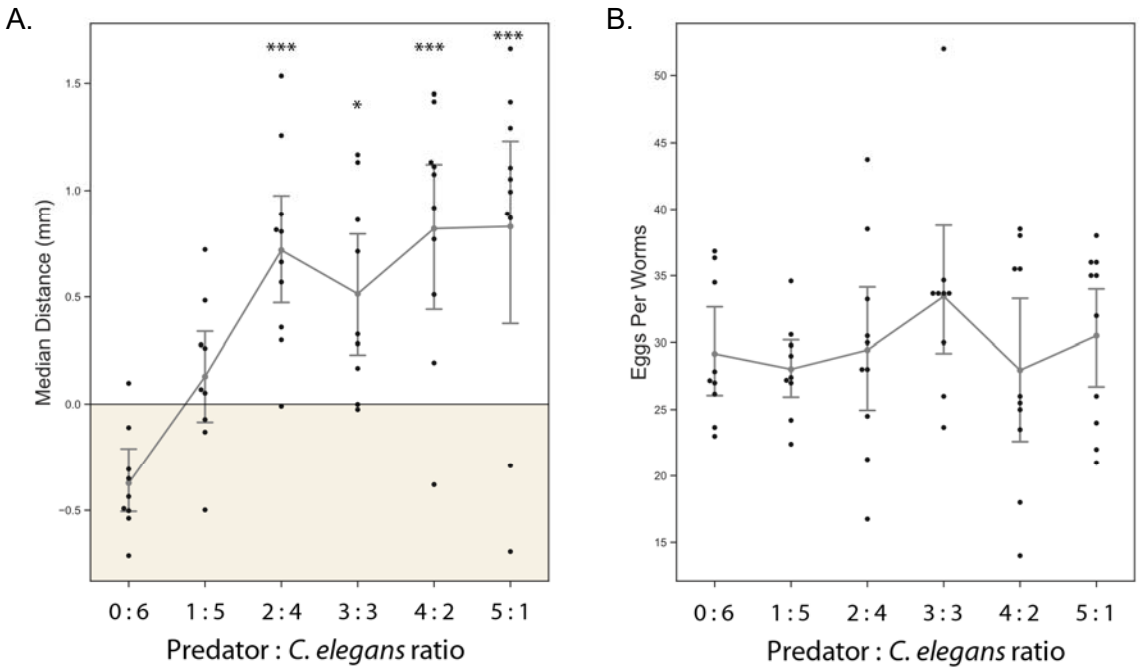


Figure 2.6 Influence of predator ratio on egg distribution behavior, and number of eggs laid. Significance of interaction between predator ratio and measurement (median distance or eggs per worm) analyzed using a one-way ANOVA. Error bars represent 95% CI. **A.** Predator ratio has an effect on egg distributions (p=9E-7). Pairwise comparisons conducted using Tukey's HSD post-hoc test, significant differences between no predator and each other ratio is shown. * p<0.05, ** p<0.005, ***p<0.0005. **B.** Predator ratio does not affect eggs laid (one-way ANOVA, p=0.55).

Egg location vs. body location

The change in egg location could be a result of multiple behavioral strategies. Normally, *C. elegans* lays its eggs on food. It is possible that *C. elegans* might lay eggs away from food but still stay near the food itself. This would decouple location from egg-laying position. This kind of opposing preference for position versus oviposition has been demonstrated in *Drosophila* [39]. The other possibility is that the *C. elegans* itself spends more time away from the lawn. If this is the case, then location of the animal itself should match the spatial distribution of eggs. I hypothesized that egg location could be used as a proxy for the location of the animal's body itself, essentially functioning as an endpoint location tracker. This would mean that the *C. elegans* body location should match egg location.

To test whether location of the animal's body corresponded to the location of the eggs, I used a device called the WormWatcher (refer to Methods) to image an array of arenas with or without predators over the 20-hour assay time. To distinguish between *C. elegans* and *P. uniformis*, I used a whole-body fluorescent strain ARM112 [40]. Using the images of fluorescent *C. elegans*, I determined their location relative to the lawn. Predator-exposed *C. elegans* started to diverge from the *C. elegans*-only control condition at around five hours and was found off the lawn starting after the eight-hour mark (Figure 2.7). Because the *C. elegans* in this assay start as juveniles and do not begin laying eggs until eight to ten hours into the assay, the egg-laying period happens after the worm location has changed to being primarily off-lawn. This is consistent with the hypothesis that the eggs are off-lawn because the entire animal is spending more time off-lawn. This suggests that the change in egg position corresponds to a change in location. However, it is still possible that *C. elegans* egg-laying circuit itself is being altered to allow to more egg-laying in low food conditions.

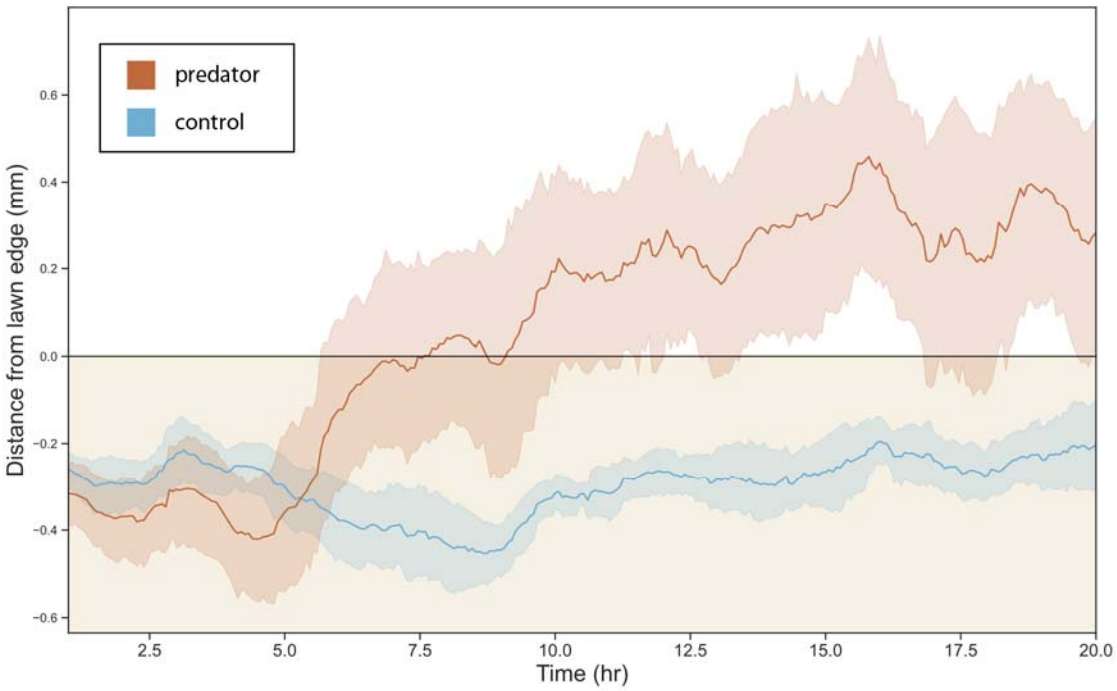


Figure 2.7 Location traces of fluorescent *C. elegans* with or without a predator present over the course of the twenty-hour assay. Images are acquired every four minutes and distances from each worm's midpoint to the lawn edge is calculated. Data was then smoothed using a rolling average with an hourly bin. Line represents mean, shaded portion is 95% CI. n= 12 wells per condition.

Chemical conditioning

We have previously shown that *P. pacificus* secretes sulfolipids that induce avoidance in *C. elegans* [32]. It is possible that *P. uniformis* could also secrete some aversive chemicals while crawling on the assay plate, which causes the *C. elegans* to avoid the lawn. To test this, I picked either *P. uniformis* or sterile *C. elegans* (to simulate the lawn disturbance caused by worm movement) to an assay plate and let them condition it overnight. After removing the conditioning worms, I moved naïve gravid *C. elegans* to the conditioned lawns and determined their egg distribution after two hours of egg-laying (Figure 2.8). There was no difference between *P. uniformis*-conditioned assay plates and *C. elegans*-conditioned assay plates. These results indicate that *P. uniformis* does not secrete aversive chemicals that account for the egg distribution change seen in the overnight assay.

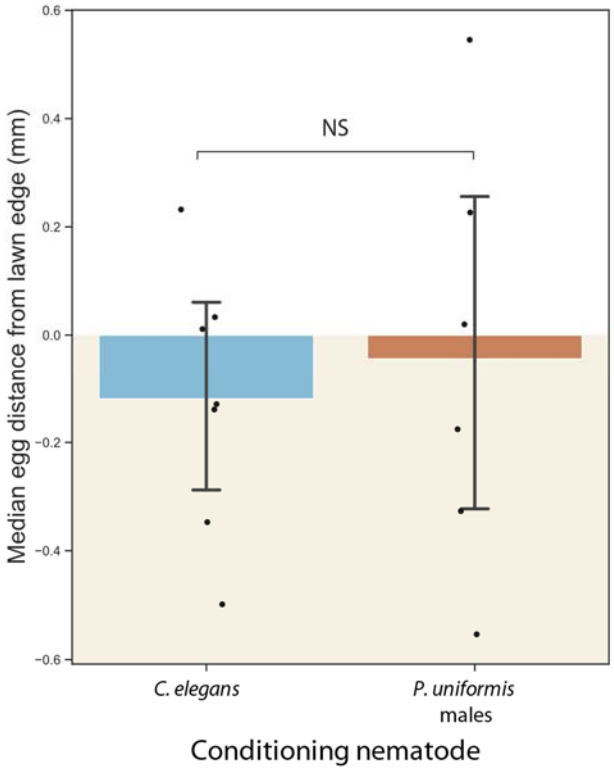


Figure 2.8 *P. uniformis*-conditioned lawns do not induce more avoidance than *C. elegans*-conditioned lawns. NS = p>0.05, Welch's t-test.

2.2. Biogenic amines

Biogenic amines are known to modulate behaviors over long time scales, and this behavior takes over six hours to take effect, and appears to last for many hours. I hypothesized that biogenic amines may play a role in the egg-laying distribution change.

Dopamine synthesis is required

To investigate the involvement of biogenic amines, I selected several mutants deficient in biogenic amine synthesis and signaling. The mutants I tested were: *cat-1*(e1111), the *C. elegans* homolog for the mammalian vesicular monoamine transporter (VMATs) [41]; *cat-2*(e1112), which encodes tyrosine hydroxylase for dopamine synthesis [42], [43]; *tdc-1*(n3419), tyrosine decarboxylase for tyramine synthesis [44]; *tph-1*(mg280), tryptophan hydroxylase for serotonin synthesis [45]; and tbh-1(n3247), tyramine beta-hydroxylase for octopamine synthesis [44].

Out of these mutants, the *cat-1*, *cat-2*, and *tph-1* mutants showed defects in the ability to change their egg distribution behavior in response to predators (Figure 2.9 A). The *cat-1* mutant disrupts all biogenic amine signaling, confirming a role for biogenic amines in general. The *cat-2* and *tph-1* mutant behavior indicated that dopamine and serotonin played roles in this behavior, but tyramine and octopamine are not involved. I decided to focus on the role of dopamine in this behavior, as serotonin-deficient mutants are already defective in egg-laying, which is the main behavioral output of the egg distribution assay. A second *cat-2* mutant allele *n4547* was also defective in responding to predator, confirming the importance of the *cat-2* gene (Figure 2.9 B).

CAT-2 is expressed in eight neurons in: four CEPs, two ADEs, and two PDEs [42]. Using promoter fragments, we drove expression of *cat-2* cDNA either in CEP (p27 promoter)

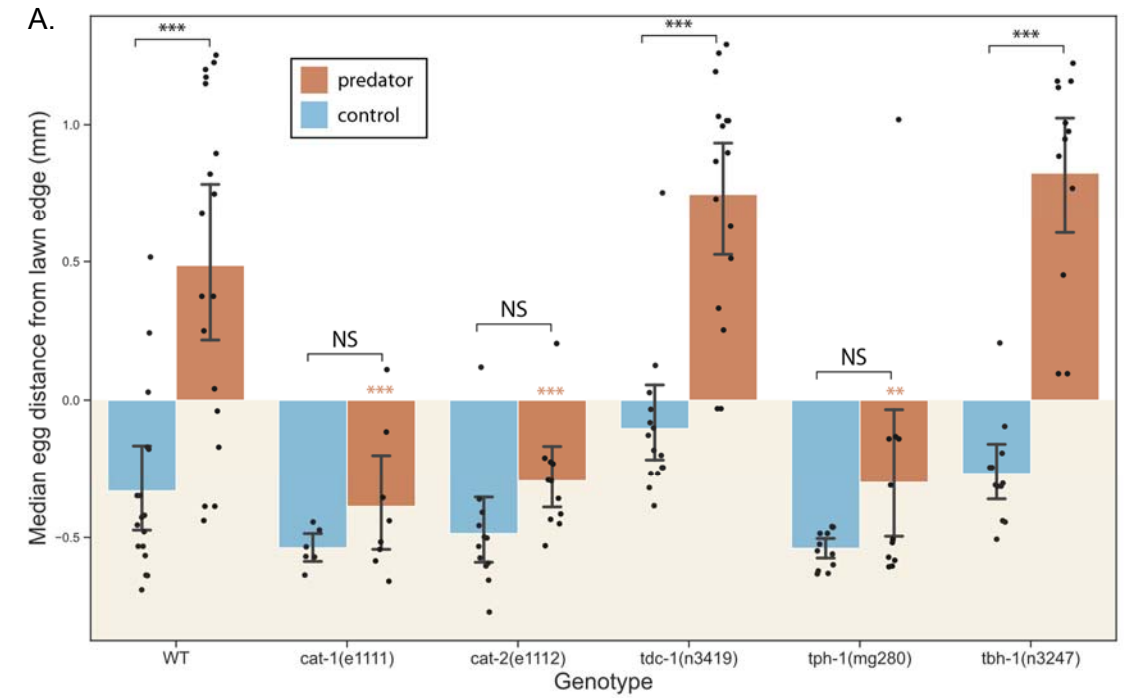
or ADE/PDE (dat-1 p19 promoter) [46]. Both rescues were able to restore response to predator but only the CEP rescue increased off-lawn egg-laying to near wild-type levels (Figure 2.9 C). These results indicate that dopamine synthesis in CEPs plays a large role in *C. elegans*' ability to modulate their egg distribution in response to predator presence, and dopamine synthesis in ADE/PDE may play a minor role.

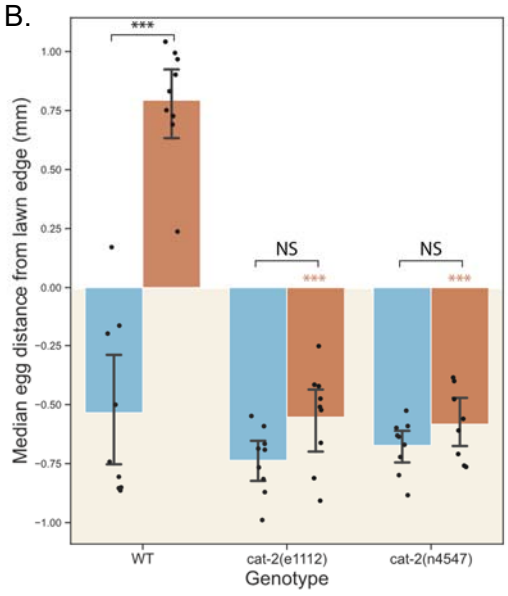
Next, I hypothesized that the *cat-2* mutant does not change its location like wild-type in response to predator. To test this, I crossed the *cat-2*(*e1112*) mutation into the fluorescent background to monitor their location in the WormWatcher. Over the course of the assay, the *cat-2* mutant remains close to the lawn through the assay (Figure 2.10 A). However, there is a slight change in behavior in the predator-exposed condition that is not seen in the control *cat-2* condition. This is reflected in the egg distribution data as well, where the predator-exposed *cat-2* egg distributions trend slightly higher than the *cat-2* control egg distributions, although this difference is not detectable in statistical tests. This suggests that dopamine deficiency depresses *C. elegans* off-lawn movement, leading to less change in locomotion following predator exposure, and as a result less change in egg distribution. It is still possible that dopamine's role in regulating egg-laying is affecting this behavior. Dopamine release in PDE has been shown to increase egg laying during roaming states [47], so the loss of this coupling could also result in fewer eggs laid off the lawn increasing the severity of the egg-laying phenotype.

Since reducing the amount of available dopamine with the *cat-2* mutant depressed the locomotor response to predator, I hypothesized that increasing dopamine levels would have the opposite effect and increase the response to predator. Dopamine can signal extrasynaptically to affect locomotion [48]. Excess dopamine is normally removed by the dopamine transporter, DAT-1. Disruptions in the *C. elegans dat-1* gene accelerate

swimming-induced paralysis (SWIP)[49]. To test whether increasing available dopamine increases locomotory response to predator, I crossed the *dat-1*(*ok157*) allele into the whole-body fluorescent background strain and imaged it over time in the WormWatcher. The *dat-1* mutants indeed traveled farther away from the lawn edge than wild-type controls (Figure 2.10 B). This increase was seen in both predator and control conditions. This confirms that increasing dopamine levels chronically can increase lawn-leaving. These results suggest that dopamine is responsible for the downstream behavioral effects of predator exposure, which is consistent with its role in other behaviors to link environmental changes to locomotion changes.

Figure 2.9 Dopamine synthesis is required for egg distribution change in response to predator. **A.** A candidate screen of biogenic amine-relate genes. **B.** A second *cat-2* allele *n4547* shows a similar phenotype to *e1112*. **C.** Transgenic rescue of *cat-2* using a CEP-expressing promoter p27 or ADE/PDE-expressing promoter dat-1 p19 [46] show differing degrees of rescue in a *cat-2(e1112)* background. Error bars represent 95% confidence interval. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between N2/WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant differences between pairwise comparisons are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.


A.
predator
control
Median egg distance from lawn edge (mm)
1.0
0.5
0.0
−0.5
***
NS
***
NS
***
NS
**
***
WT
cat-1(e1111)
cat-2(e1112)
tdc-1(n3419)
tph-1(mg280)
tbh-1(n3247)
Genotype


B.
Median egg distance from lawn edge (mm)
1.00
0.75
0.50
0.25
0.00
−0.25
−0.50
−0.75
−1.00
***
NS
***
NS
***
WT
cat-2(e1112)
cat-2(n4547)
Genotype

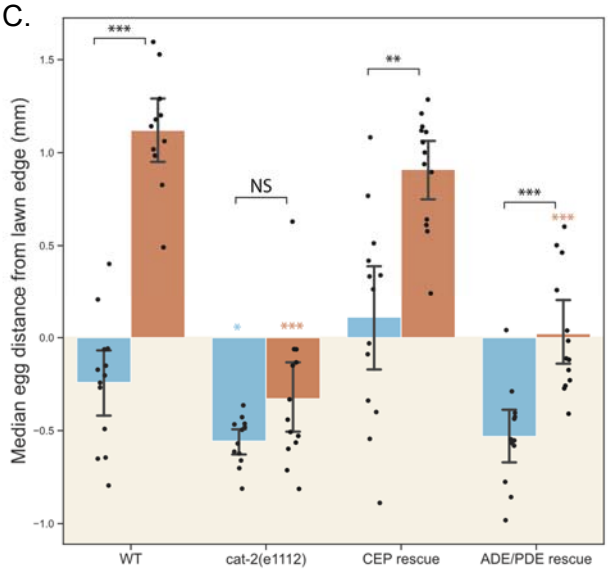
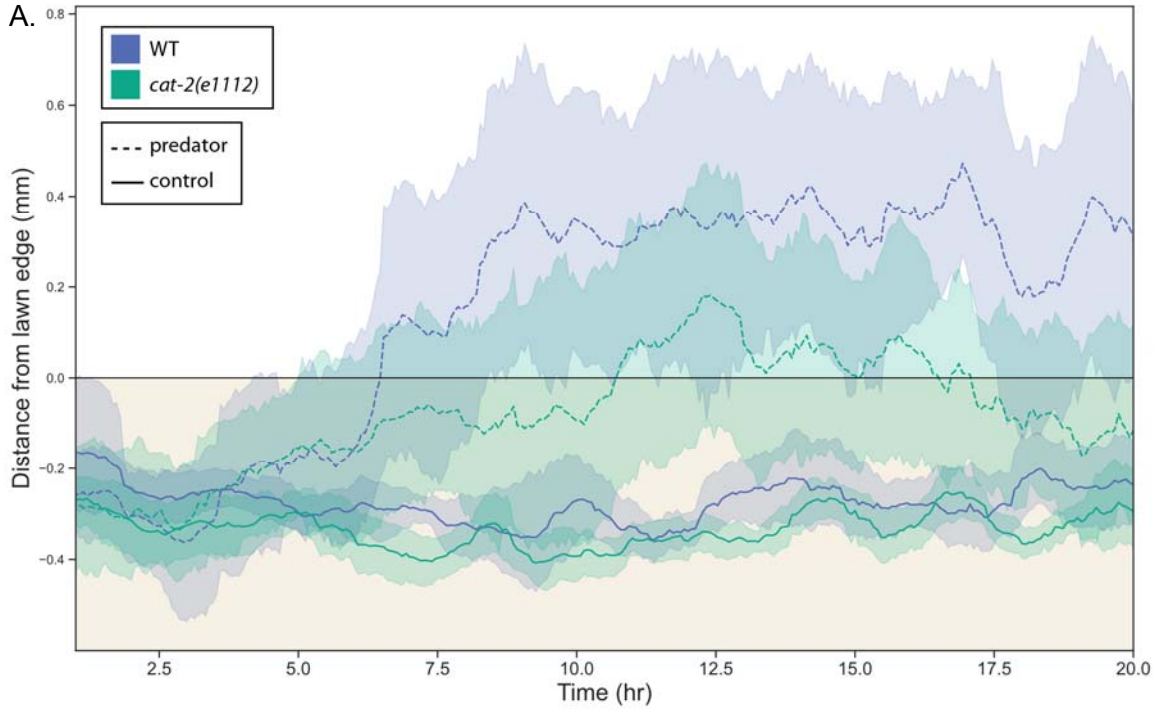
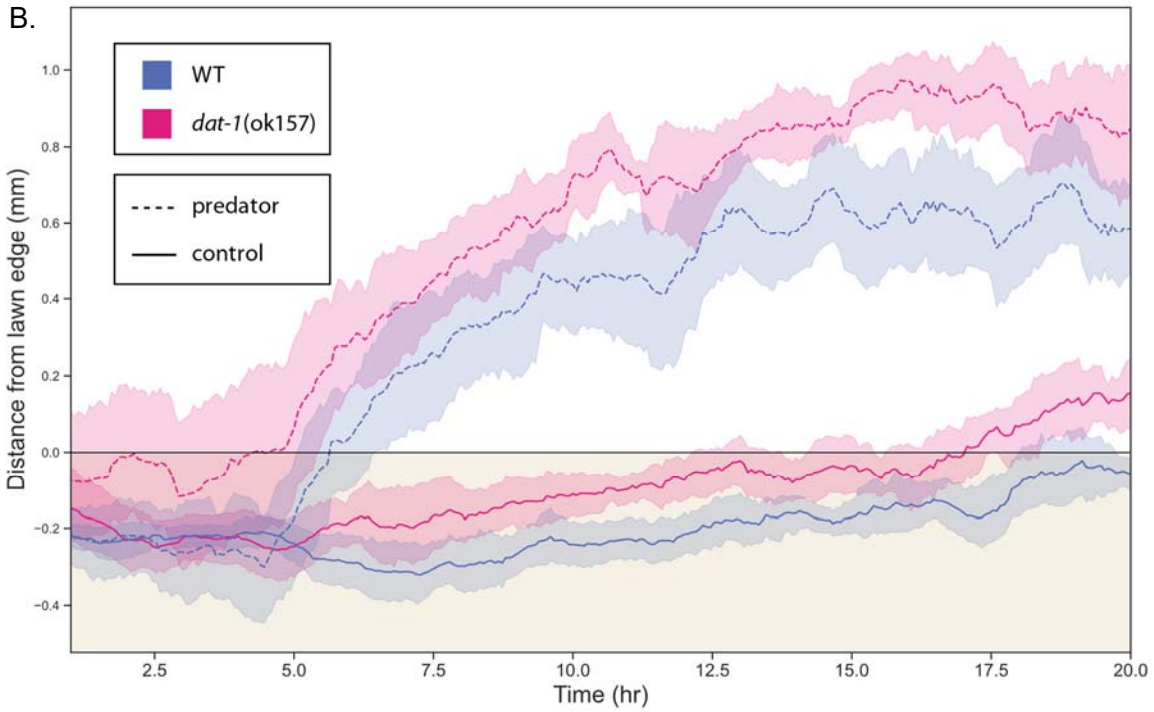

C.
Median egg distance from lawn edge (mm)
1.5
1.0
0.5
0.0
−0.5
−1.0
***
NS
**
***
*
***
***
WT
cat-2(e1112)
CEP rescue
ADE/PDE rescue

Figure 2.10 Location traces of *cat-2* or *dat-1 C. elegans* with or without a predator present over the course of the twenty-hour assay. **A.** Fluorescent *C. elegans* carrying the cat-2(e1112) allele compared to wild-type. **B.** Fluorescent *C. elegans* carrying the *dat-1(ok157)* allele compared to wild-type. Dashed lines represent the predator-exposed condition, unbroken lines represent no-predator controls. Images are acquired every four minutes and distances from each worm's midpoint to the lawn edge is calculated. Data was then smoothed using a rolling average with an hourly bin. Line represents mean, shaded portion is 95% CI. n= 8-12 wells per condition.


A.
WT
cat-2(e1112)
predator
control
Distance from lawn edge (mm)
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (hr)


B.
WT
dat-1(ok157)
predator
control
Distance from lawn edge (mm)
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (hr)

Multiple dopamine receptors are involved

There are at least four dopamine receptors in *C. elegans*: *dop-1*, *dop-2*, *dop-3*, and *dop-4*. To determine which receptor(s) may be receiving the dopamine signals, I tested single mutants in each of these receptors, but none of the single mutants had detectably different behavior than wild-type (Figure 2.11) To test whether a combination of receptors may need to be involved to show an effect, I tested double mutants in every possible combination of the four receptors (Figure 2.12 A). Each double mutant was able to alter its egg distribution behavior in response to predator, but there was a decrease in the *dop-1, dop-3* double mutant response to predator. Additionally, the *dop-1, dop-2* double mutant had a control egg distribution close to the edge than wild-type and *dop-3, dop-4* had a control egg distribution closer to the center of the lawn than wild-type. This suggested that inherent, and opposing, effects of the dopamine receptors on egg distributions could be affecting or masking the effect of predator. Specifically, while *dop-1* combined with *dop-3* could drive some of the predator-induced increased off-lawn egg-laying behavior, *dop-2* combined with *dop-1* increased the baseline tendency to lay eggs away from the center of the lawn while combining *dop-4* with d*op-3* drove the opposite. To test whether combining the *dop-2* mutation with the *dop-1, dop-3* mutants increased off-lawn egg-laying, I tested a triple mutant in *dop-1, dop-2*, and *dop-3* as well as the quadruple mutant and found that they both displayed strongly reduced off-lawn egg-laying behavior, although they still retained some sensitivity to predator presence (Fig 2.12 B). Interestingly, the quadruple mutant appears to show a more variable egg distribution behavior, possibly reflecting the influence seen in the double mutants of *dop-3*, *dop-4* on driving basal egg-laying closer to the center of the lawn. Therefore, *dop-1, dop-2*, and *dop-3* combine to drive the egg distribution behavior change in response to predator, and *dop-4* is either dispensable to this phenotype or drives the opposite behavior.

While the dopamine receptor triple and quadruple mutants showed reduced off-lawn egg-laying, they were not able to completely abolish the change in behavior as seen in the *cat-2* mutant. This suggests that there may be additional pathways through which *cat-2* modulates this behavior. These pathways could include additional dopamine receptors or developmental abnormalities present in CAT-2-deficient animals but not present in the receptor-deficient animals.

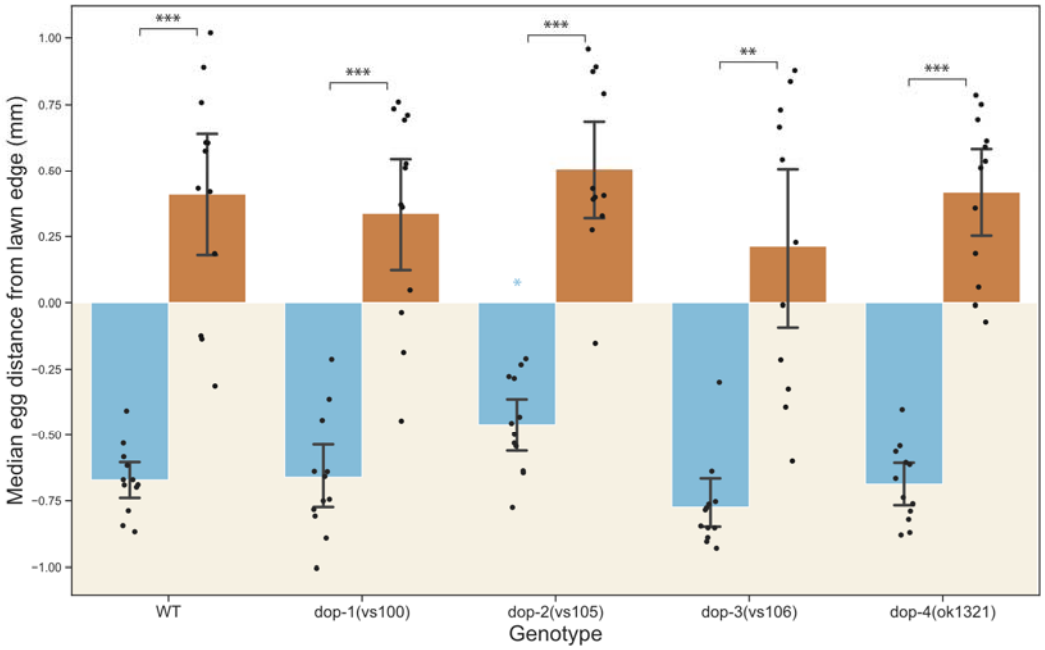
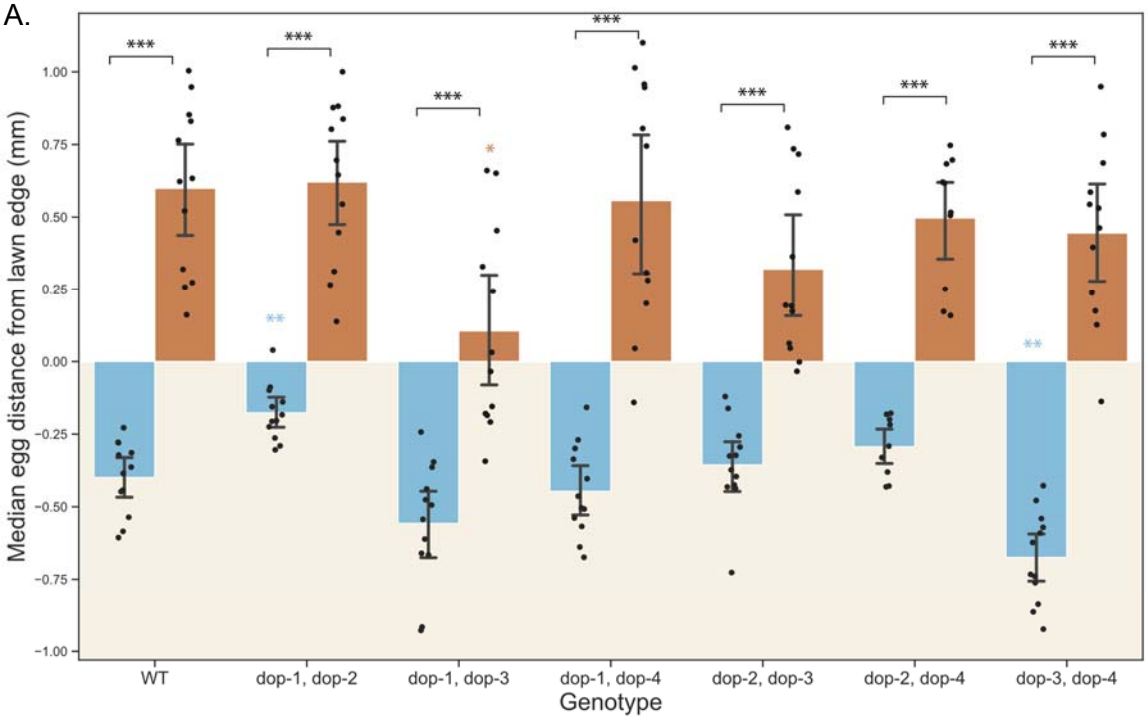


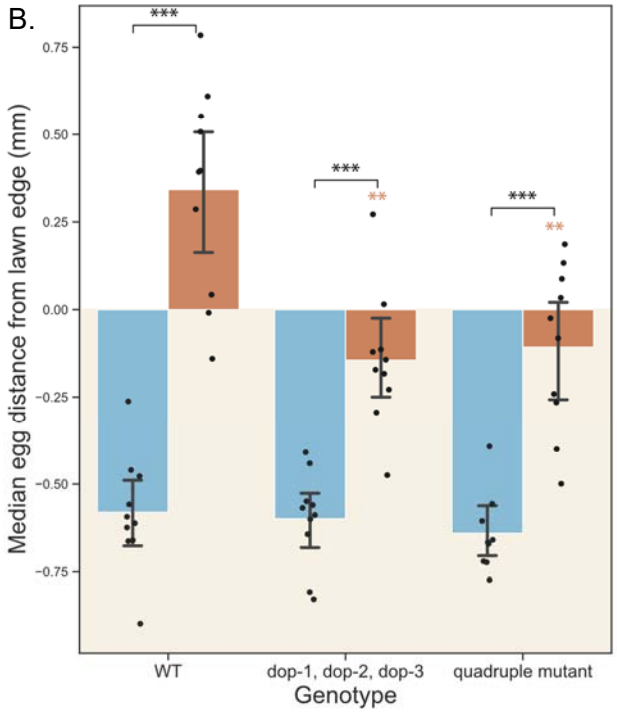
Figure 2.11 Single mutants of dopamine receptors are still sensitive to predator exposure. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant pairwise differences are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

Figure 2.12 Multiple dopamine receptors modulate egg distribution change in response to predator. **A**. Double mutants in every combination between *dop-1*, *dop-2*, *dop-3,* and *dop-4*. **B**. A triple mutant without *dop-4* and a quadruple mutant in all four dopamine receptors. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant pairwise differences are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

A.


Median egg distance from lawn edge (mm)
1.00
0.75
0.50
0.25
0.00
−0.25
−0.50
−0.75
−1.00
***
**
*
WT
dop-1, dop-2
dop-1, dop-3
dop-1, dop-4
dop-2, dop-3
dop-2, dop-4
dop-3, dop-4
Genotype

B.


Median egg distance from lawn edge (mm)
0.75
0.50
0.25
0.00
−0.25
−0.50
−0.75
***
**
WT
dop-1, dop-2, dop-3
quadruple mutant
Genotype

2.3. Effect of predator on foraging

To effect a change in location relative to the dense food patch in the center of the assay plate, *C. elegans* may be using some aspects of its “landscape” to navigate. One noticeable aspect of the assay plate post-interaction is that on the control plates without predator, the bare agar outside the lawn remains relatively bare still. However, on the plates with predator added, streaks of bacteria form in the outside area. These streaks are inevitable in a long assay with animals leaving the lawn, because every exit can drag a small amount of bacteria outside the original lawn and over the long assay time, the bacteria streak can then grow. This streaking out of the bacteria creates an area where *C. elegans* can still eat, though food is less abundant.

Egg distribution change is not only due to change in bacterial “landscape”

To test whether the presence of streaks alone could account for the change in egg distribution, I created artificially smeared lawns by dragging the tip of an eyelash pick through the dense lawn and out onto the bare agar. The artificial smears grow over time until by the end of the assay there are visible bacteria smears though the dense bacteria patch in the center remains intact, as happens in the predator condition (Figure 2.13 A). When comparing non-smeared lawns containing only *C. elegans* and the artificially smeared lawns containing only *C. elegans*, the artificial smears induce slightly more off-lawn egg-laying. However, *C. elegans* exposed to predator still lay more eggs off-lawn than *C. elegans* only on artificially smeared lawns (Figure 2.13 B). Therefore, the presence of bacteria smears outside the lawn is not sufficient to drive the magnitude of the change in egg distribution behavior when a predator is present.

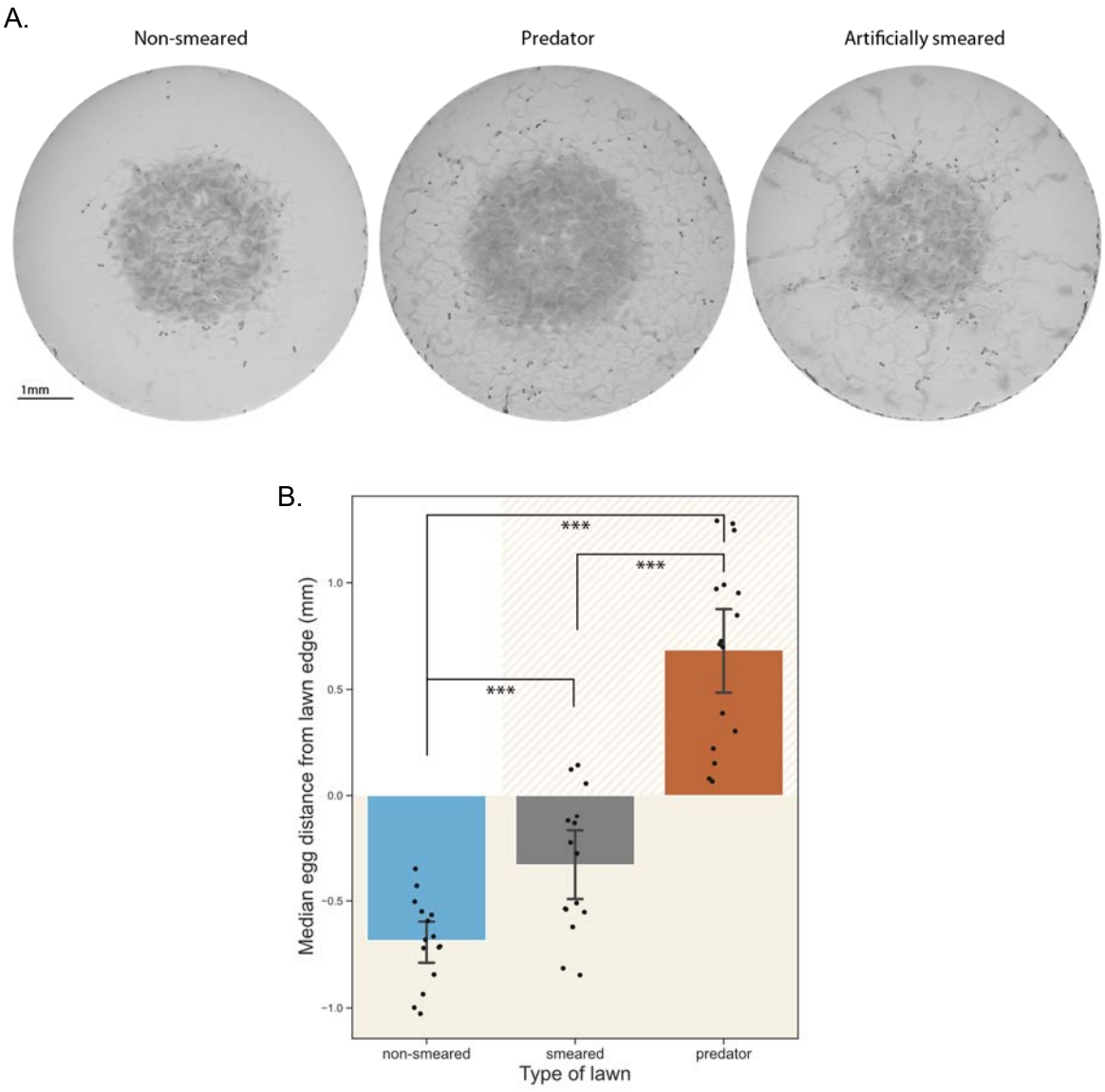


Figure 2.13 Smearing the edge of the bacterial lawn is not sufficient to account for the change in egg distribution in predator-exposed animals. **A.** Images of lawn types at the end of the twenty-hour assay. **B.** Egg distribution does change in the artificially smeared lawn, but not as much as in lawns with predator. Significance determined with a one-way ANOVA followed by Tukey's HSD for pairwise comparisons. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

Egg distribution change lasts for hours without predator

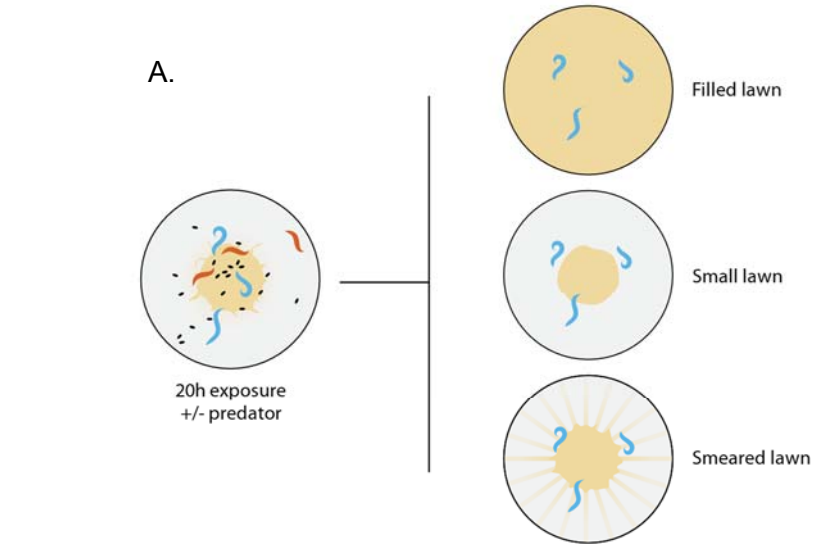
Next, I wanted to test whether the change in egg distribution behavior could persist even without predators, and how this related to presence of bacteria. I "trained" *C. elegans* in the overnight assay with predators and then transferred only the *C. elegans* to three different kinds of test plates: a filled plate, where the entire zone inside the corral was evenly covered in food, a small lawn, which had the same small lawn in the center of the corral and bare agar around it, and the smeared lawn, which was created using the same method as in Figure 2.13. I then imaged the plate every hour for six hours (Figure 2.14 A). If predator exposure altered egg distribution in the filled lawn, then predator-exposed *C. elegans* may be using the edge of the corral to navigate, rather than bacteria. If egg distribution on the small lawn is altered, then *C. elegans* may be avoiding food altogether. If egg distribution on the smeared lawn is altered, then *C. elegans* may either be preferring the lower-density food zone itself, or at least not preferring the dense patch over the smears themselves as un-exposed *C. elegans* do.

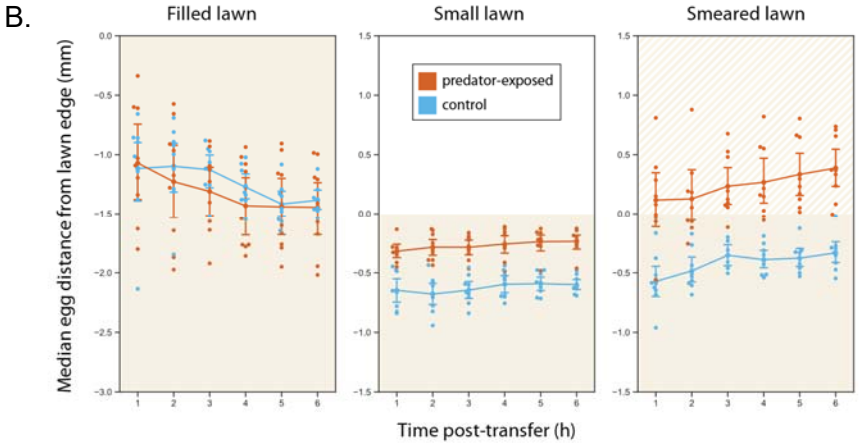
As shown in Figure 2.14 B, predator exposure does not alter egg distribution on the filled lawn, indicating that predator-exposed and control animals disperse themselves similarly on bacteria alone, and they are not using the corral edge to navigate. On the small lawn, the predator-exposed animals have a slightly increased median egg distance, though the median remains negative indicating that the median still lies within the lawn boundary. Looking at the distribution of each egg in Figure 2.14 C, most eggs are laid inside the lawn but close to the edge. Eggs laid off-lawn are a few eggs which can be quite far from the lawn boundary, suggesting that overall the predator-exposed *C. elegans* prefer food to no food, though they will exit the lawn and lay eggs more often than control *C. elegans*. In the case of the smeared lawn, the median egg distance for predator-exposed *C. elegans* moves into the positive, off-lawn region. Observing the distribution of each egg again, the predator-exposed

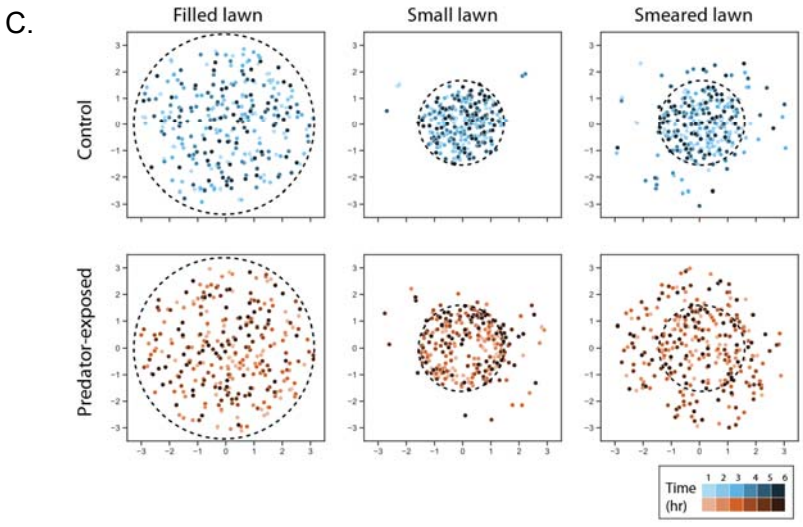
C. elegans do not avoid the middle lawn but instead lay eggs throughout the entire assay plate, in both the dense lawn and the zone with only bacteria smears.

Interestingly, this altered egg-laying behavior persists throughout the entire observed six hours and does not appear to show any decline, despite the lack of predator. It is possible that some specific aspects of the environment might be reinforcing the behavior and allowing it to persist for so long. As covered in Chapter 1, fear learning behaviors can persist for over a day if the training sessions are repeated. It remains to be tested how long this change in foraging behavior persists.

Figure 2.14 Long-term foraging strategy is altered in predator-exposed animals. **A.** Experimental setup for assay showing the different types of food environments. **B.** Egg distribution remains changed for at least six hours in the “small” and “smeared” lawn types. Error bars represent 95% CI. **C**. Sample egg distributions relative to the food (dashed line circle). 50 eggs were randomly sampled and plotted from each time point to create representative plots.


A.
Filled lawn
Small lawn
Smeared lawn
20h exposure
+/- predator


B.
Filled lawn
Small lawn
Smeared lawn
predator-exposed
control
Median egg distance from lawn edge (mm)
Time post-transfer (h)


C.
Filled lawn
Small lawn
Smeared lawn
Control
Predator-exposed
Time (hr)

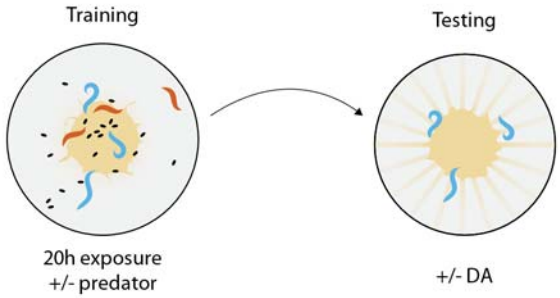
2.4. Exogenous dopamine affects predator-exposed animals

Next, I wanted to find out when dopamine acts in this assay. Based on the location data from the *cat-2* and *dat-1* mutants, I hypothesized that dopamine release in the latter portion of the assay was required for execution of the behavior. If this were the case, then exogenous dopamine should be able to rescue the behavior of *cat-2* mutants after predator exposure. To test this hypothesis, I trained N2 or *cat-2* mutants in the usual assay with or without predators and added exogenous dopamine to the smeared lawn test plates (Figure 2.15 A). 2mM dopamine, which has been shown to rescue basal slowing phenotypes [26] and density pattern discrimination phenotypes [50], was used in these rescue experiments.

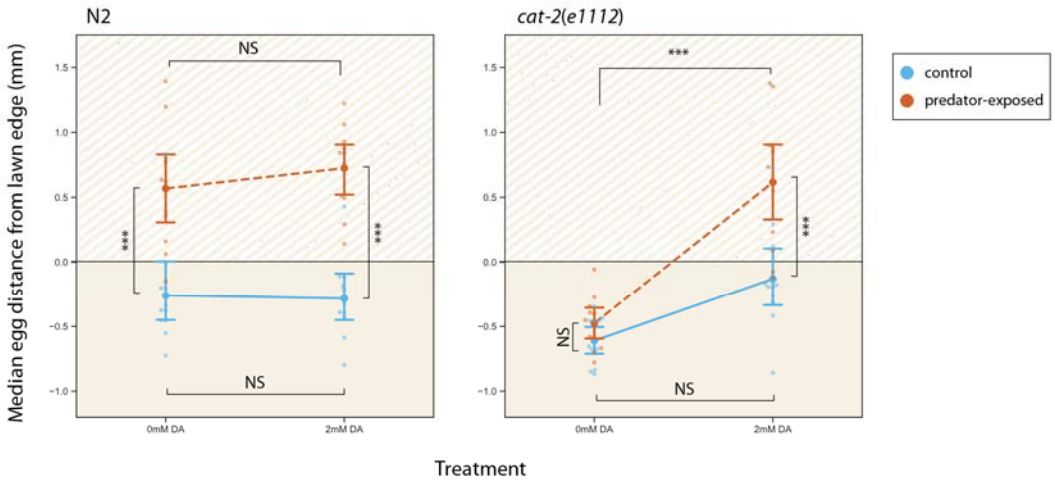
In these experiments, exogenous dopamine had no effect on wild-type N2 animals, though the effect of predator on inducing more off-lawn egg laying was intact. In the dopamine deficient *cat-2* animals however, untreated animals were unaffected by predator exposure while dopamine treatment differentially affected predator-exposed *cat-2* animals, inducing off-lawn egg-laying similar to wildtype levels (Figure 2.15 B, C). This confirmed my hypothesis that predator exposure requires dopamine for execution of long-term off-lawn egg laying behavior.

Figure 2.15 Exogenous dopamine rescues learning defect in *cat-2* mutants, and its effect is differential depending on past predator experience. **A.** Assay setup. Training occurs with or without predator for twenty hours, then the trained *C. elegans* are transferred to a smeared lawn test plate with or without 2mM dopamine. **B.** Egg distribution is affected by predator only in wild-type N2; adding dopamine to the test plate rescues this in the *cat-2* mutant. The effect of exogenous dopamine is only detectable in the predator-exposed *cat-2* mutants. A three-way ANOVA showed a significant interaction ($p<0.0.5$) between two of each possible category (strain, dopamine treatment, predator exposure) but failed to find a significant interaction between all three. Significant differences between pairwise comparisons were tested using using Tukey's HSD and relevant interactions are shown. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$. **C.** Representative plots of the egg positions relative to the lawn (dashed line circle). 150 eggs were randomly sampled from each condition for each plot so that 300 eggs total are shown in each plot.

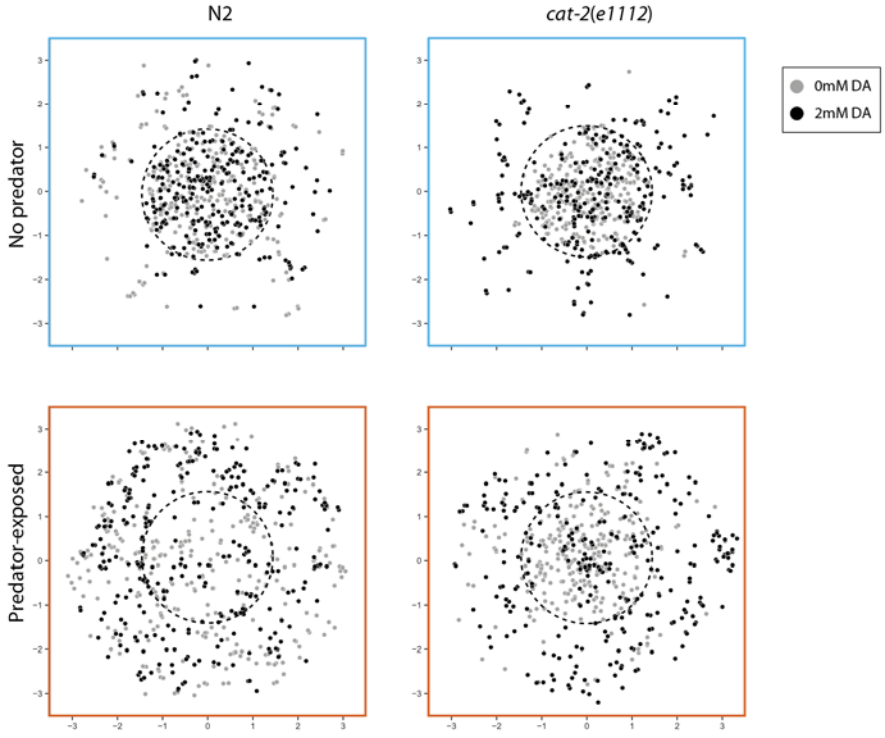
A.


Training
Testing
20h exposure
+/- predator
+/- DA

B.


N2
cat-2(e1112)
Median egg distance from lawn edge (mm)
NS
***
NS
control
predator-exposed
0mM DA
2mM DA
Treatment

C.


N2
cat-2(e1112)
No predator
Predator-exposed
0mM DA
2mM DA

3. Methods

Egg distribution assay

Assay plates are created by spotting 0.5ul of OP50 (OD_{600}=0.5) on 35mm NGM plates. The bacterial lawns are allowed to grow at 20°C for 30 hours, then stored for up to one month at 4°C. Whatmann filter paper with ¼" punch forms the "corral" and encircles the lawn, allowing approximately 1.5mm of clean agar in between the lawn edge and the corral edge. All animals are allowed to crawl on a clean section of agar to clean them of bacteria and picked to the assay plate using a sanitized eyelash, placed next to the lawn on a clean area of agar. Three predators are picked first, staged by overall size and pigment development as J4s. Then three *C. elegans* L4s are picked to the assay plate. The animals are allowed to interact for a determined amount of time, 20 hours for an overnight assay, at 20°C. For short-term exposure (6 hours and under), gravid *C. elegans* adults and adult predators are used by picking L4s or J4s the day before to plates with plenty of food. The juveniles are allowed to grow overnight into adulthood and then used in the same assay setup. After their interaction, corrals and all adults are removed from the plate and the area inside the corral is imaged using a ZEISS AxioZoom V.16.

For the smeared lawn variation, smears are formed by gently dragging a sanitized eyelash through the center of the lawn in radial streaks ten times, followed by a two concentric circular streaks halfway between the lawn and the corral edge.

Injury assay

Injury assays are set up in the same way as the egg distribution assays, using a *C. elegans* strain containing the array frIs7 [nlp-29p::GFP + col-12p::DsRed]. After the set interaction time, worms are immobilized by placing the plates on ice and imaged **(**ZEISS

AxioZoom V.16) within one hour, with exposure times kept constant for fluorescence imaging (25ms)**.** Assays are performed with their relevant controls over at least three separate days.

Learning assay

C. elegans are trained using the overnight egg distribution assay. At the same time as the animals used for training are transferred to their assay plates, test plates are set up. Three types of test plates are used: a filled lawn (10ul of OP50 (OD_{600}=0.5), a smeared lawn (same as the smeared lawn variant of the egg distribution assay), and a small lawn (same as the original assay plate). The training plates with animals on them and the test plates are incubated at 20°C for 20 hours, during which the *C. elegans* is exposed to JU1051 males and the smears on the test plates are allowed to grow. The bacteria on the other test plates is also allowed to grow at this time so that the bacteria is at a similar metabolic state and density across test plates. Filter paper corrals like those used in the egg distribution assay are centered over the test plate lawns.

After the *C. elegans* are incubated in their training conditions for 20 hours, they are carefully removed with an eyelash pick from their training plates to a clean section of an NGM plate. The animals is allowed to crawl for a few seconds to remove bacteria and then picked to a test plate halfway between the central lawn and the corral edge. For the filled test lawns, the animals are placed in an equivalent position relative to the corral edge. Three *C. elegans* are transferred to each test plate. The test plates are then imaged every hour on an AxioZoom V.16 for 6 hours.

Exogenous dopamine assay

When adding exogenous dopamine to the learning assay, a 200mM stock of dopamine hydrochloride (Code 122000100 Lot: A0427132, CAS: 62-31-7, Acros Organics) in water was prepared. Two hours before the trained worms needed to be transferred to the

test plates, 50µl of the dopamine stock or water as a control was dropped onto the test plate, mostly next to the test plate lawn. The plates were allowed to diffuse and dry with the lids off for two hours, at which time the trained worms were transferred to the test plates. The trained worms were allowed to lay eggs for two hours before their plates were imaged. The exogenous dopamine assay used only the smeared lawn type of test plate, because this helped to exaggerate the predator-exposed behavior and better reflected the actual state of the training plate at the end of the training period.

Egg distribution image quantification

Egg distribution images are quantified in FIJI with the experimenter blinded to the condition by randomizing the file order and obscuring the filenames (using the Filename_Randomizer macro found at https://imagej.nih.gov/ij/macros/Filename_Randomizer.txt). Eggs are manually selected with the multipoint tool and lawns are selected are circles. Distances from each egg from to the lawn edge are calculated in Python. Assays are performed with their relevant controls over at least three separate days.

WormWatcher assays

Assays conducted in the WormWatcher (Tau Scientific Instruments) were performed on a single 6cm 2.5% agar NGM plate in a 12-arena setup. The 12-well arena was created by cutting a 3x4 array of ¼” circles into a plastic sheet using a Cricut machine. OP50 was spotted in the 3x4 pattern using the same concentration and allowed to grow for the same amount of time as in the egg distribution assay. The increased agar percentage on the WormWatcher plates helped prevent worms from escaping under the plastic edges of the arenas.

The assays were set up like the egg distribution assays, with three L4 *C. elegans* and three J4 JU1051 males or six L4 *C. elegans* in control wells. The positions of predator-containing and/or mutant-containing wells was alternated on different assay days. The WormWatcher was set to acquire fluorescent frames with a green LED excitation light every four minutes. A reference darkfield image was acquired before and after every experiment to reference the positions of the arenas and the size and positions of the lawns. After the experiment was completed, each area was inspected and image to determine whether any worms escaped away or into it. Custom code was written to segment the worms and wells in each position and the median distance to the mid-point of each worm body per well was recorded. These distance measurements were smoothed with a rolling average by hour. Data from arenas were discarded if two worms had escaped from an arena, or if a *P. uniformis* was seen in a control arena.

Pristionchus mouthform analysis

Pristionchus mouthform analysis was performed as reported in [20]. Briefly, *Pristionchus* were egg-prepped via bleaching and eggs were either cultured on standard solid NGM plates or in liquid culture. After eggs reached adulthood, they were immobilized on agarose slides with sodium azide. The slides of different strains from different culture conditions were mixed and their labels obscured while they were observed. The slides were scored as either Eu (wide mouth, two teeth) or St (narrow mouth, one tooth) while the experimenter was blinded.

4. Conclusions

In the above experiments, I have demonstrated that *C. elegans* can be used as a model to study prey-predator interactions using *Pristionchus* as a predator. As in most other predator-prey interactions, *P. uniformis* predators alter *C. elegans* behaviors by altering its foraging behavior, making it stay away and law eggs far from a dense patch of food. This change in foraging behavior persists for at least six hours.

4.1. Predator exposure alters foraging preference

At the beginning of the assay, *C. elegans* experiences bites from the predators. Since a predator with the Eu mouthform is required to change *C. elegans* behavior, the experience of being bitten is required. These bites are probably mostly experienced while on the dense patch of food in the center of the arena, because *Pristionchus* prefers to stay on bacterial food [33]. *C. elegans* that receive a bite will conduct a reversal (or rapidly move forward, depending on the location of the touch). These reversals can lead to lawn-leaving events. While leaving the food, the body of the animal will drag a small amount of bacteria outside the lawn and over time these will grow into bacterial smears that are a not preferable, but not insignificant, source of food. At around six hours of predator exposure, *C. elegans* switches preferences to this outside area over the dense lawn, staying off the main lawn and laying eggs away from it.

After twenty hours in this environment with a predator, if transferred to a new arena, *C. elegans* continues to exhibit shifted foraging behavior for at least six hours. This shows that *C. elegans* has learned about predator presence and can exhibit learned behavior in the absence of predators. In the case of the new arena with a defined patch of food and bare agar, the *C. elegans* will stay close to the edge of the food rather than dispersing throughout it and will also leave the lawn relatively often and lay eggs on bare agar. In the case of a new

arena with bacterial smears outside it, the predator-exposed *C. elegans* will lay eggs in the smeared bacteria more often rather than preferring the dense patch in the center. This behavior – the altered egg distribution with predators, and the altered egg distribution when transferred away from predators – is dependent on dopamine synthesis through CAT-2. The defect in *cat-2* mutants can be rescued by exogenous dopamine, and this rescue is most prominent in predator-exposed *cat-2* mutants rather than *cat-2* mutants that have not experienced predators.

Considering this behavioral paradigm in the context of fear learning, *C. elegans* takes the first six hours of the assay to learn about the environment and the about the predators. From hours six to twenty, *C. elegans* shifts its foraging strategy in a way that probably protects it from bites even if they may not be occupying a favored environment. Transferring the *C. elegans* to a new environment shows that the *C. elegans* can continue to perform the behavior without reinforcement from biting. This memory lasts for at least six hours. This long-term memory is not unsurprising given the long training period; *C. elegans* can remember aversive conditioning with spaced training for 24 hours [51].

4.2. Relevance of this behavior

Staying on food is important for the health of both the *C. elegans* mother as well as its progeny. *C. elegans* also can evaluate food quality and will normally choose to stay on high quality food [52]. Starvation has a myriad of effects on and adult, including low brood size, matricide due to bagging, and decreased lifespan [53]. If eggs are laid in the absence of food, the L1s that hatch may develop into the dauer stage. While the size of the arena used in these experiments would not lead to dauer formation [35], *C. elegans* still has a strong drive to stay near food and lay eggs on the food. Therefore, predator exposure alters a primary behavior in *C. elegans* by changing how it relates to food. In terms of risk-benefit

calculation, this altered foraging behavior could be the result of lowering the threshold of acceptable food density, or could be actually changing the value of a different type of food, i.e. streaky or highly variable food sources being viewed as more valuable.

4.3. Role of dopamine

Predator exposure induces long-lasting foraging changes that require dopamine. Exogenous dopamine added after predator exposure rescues long-term behavior change in *cat-2* mutants, suggesting that dopamine signaling is required during execution of the long-term behavior, but not during acquisition. In *C. elegans,* dopamine normally signals the presence of food through sensing the change in texture. Texture change alone leads to phenotypes like basal slowing on food [26]. TRP-4, a mechanosensory receptor expressed in dopaminergic neurons, is required for mechanoreceptor currents (MRCs) in CEPs [54], which send cilia to the nose of the animal are activated when an animal first enters food [55]. Changes in TRP-4 sensitivity to mechanical stimulus contributes to the native preference for denser textures [56], probably because denser textures mean more food. Predator exposure changes *C. elegans* preference to either the edge of the lawn, or the highly variable environment of the bacterial lawn. Both of these environments have high textural variability. In our previous study, we showed that textural contrast present at the edges of a lawn can result in more dopamine release from CEPs [57]. In this study, exogenous dopamine suppressed reorientations, increasing runs rather than reversals. Dopamine acted on ASI neurons, increasing their range of responses by increasing response to high bacteria concentrations but lowering their response to low bacteria concentrations. This showed a role for dopamine in modulating food search behaviors. In the current study, dopamine release appears to alter *C. elegans* preference for these high variability environments as *cat-2* mutants are unable to modulate their foraging/egg-laying preference. Therefore, increased

dopamine release in highly variable environments could account for the preference for the highly variable off-lawn location.

Dopamine release does not appear to be required for the acquisition of memory in the current study, as exogenous dopamine added after the acquisition period is sufficient to fully rescue *cat-2(e1112)* behavior. This implies that the predator exposure acts upstream of dopamine release. Predator exposure alters *C. elegans*' behavior upon exogenous dopamine addition in a dopamine-deficient background. However, this addition of exogenous dopamine does little to *C. elegans* that have not experienced predators. How, then, does predator exposure affect *C. elegans* in such a way that dopamine can induce this behavioral change? Due to the requirement for predator biting in the assay, it is possible that the experience of predator biting over several hours could be altering the *C. elegans* dopaminergic system by altering the response of the dopamine circuit. The changed response to dopamine could change decisions about foraging.

Dopamine signaling and pain sensation are linked. It is possible that the sensation of painful bites could alter dopamine signaling. Studies of the role of dopamine in pain have focused on the role of dopamine in analgesia [58]. For example, chronic drug use can decrease dopamine release and downregulate D2 dopamine receptor expression, potentially blunting an individual's ability to experience reward [59]. Parkinson's disease can result from dopaminergic neuron degeneration. Parkinson's disease patients who experience pain have lower pain thresholds than those that do not, but treatment with the dopamine precursor L-DOPA reduces these differences [58], [60]. Increasing dopamine levels in this hypodopaminergic background therefore has an antinociceptive effect. These pieces of evidence from studies in humans show that dopamine can reduce pain, usually through activation of D2 receptors, and conditions that decrease dopamine can lower pain

thresholds. This study may show that that the experience of pain can alter how the organism responds to dopamine.

Alternately, predator biting may be exerting its effect through some other way. For example, this could be an example of associative learning. *Pristionchus,* at least *P. pacificus*, are more likely to bite *C. elegans* while on food, causing the *C. elegans* to leave the food and allowing the *Pristionchus* to exploit the limited, shared resource [35], [61]. It is possible that the *C. elegans* learns to associate the areas with dense food with receiving bites, leading to the *C. elegans* to avoid dense food patches. As discussed in Chapter 1, interpreting food as an conditioned stimulus in a CS/US pairing is tricky, as food elicits an unconditioned response already in untrained animals. Food itself is highly valuable and drives most behavior in *C. elegans*. Therefore, the argument that predator biting trains *C. elegans* to associate dense food with biting requires further study. First, proving that this is associative rather than nonassociative learning requires further testing by breaking the pairing between dense food and biting and observing whether the change in foraging behavior still happens.

5. Future directions

This behavioral paradigm presents an interesting possibility to study the diverse roles of dopamine receptors. *dop-3* has been identified in multiple studies as responding to extrasynaptic dopamine. Interestingly, the D1-like receptor *dop-1* and D2-like *dop-3* can drive opposite effects in extrasynaptic dopamine-mediated paralysis during swimming in liquid by acting in the same motor neurons [48], while in this assay the two receptors appear to act synergistically. Instead, these data show a potential role for the D1-like *dop-4* in opposing the effect of *dop-3* in an on-agar behavior. Further studies will need to be performed to determine exactly how *dop-4* antagonizes *dop-3* and how *dop-1*, *dop-2*, and *dop-3* may cooperate. In particular, cell-specific rescues of these receptors in neurons where they

overlap may help to illuminate whether these receptors act in the same neurons, or if they perhaps even co-localize. If the hypothesis that predator exposure alters dopamine sensitivity is supported, then alterations to dopamine receptor expression could account for this difference.

6. Appendix

Table 2.1: List of strains used in Chapter 2

Strain Name	Genotype	Figure	Notes
N2	Wild-type	2.6, 2.8, 2.9, 2.11, 2.12, 2.13, 2.14, 2.15	From CGC
CX7389	kyIs392 [Pstr-2::GFP::rab-3; Pttx-3::lin-10::dsRed; Pelt-2::GFP]	2.3, 2.4	Fluorescent eggs
CZ6326	frIs7 [nlp-29p::GFP + col-12p::DsRed] IV	2.5	Injury reporter
ARM112	wamSi112 [eft-3p::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II; unc-119(ed3) III	2.7, 2.10	WT whole-body fluorescent strain
IV983	cat-2(e1112) wamSi112[eftp-3::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II	2.10 A	Created for this study
IV988	wamSi112[eftp-3::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II; dat-1(ok157) III	2.10 B	Created for this study
CB1111	cat-1(e1111) X	2.9 A	
CB1112	cat-2(e1112) II	2.9, 2.15	
MT13113	tdc(n3419) II	2.9 A	
MT15434	tph-1(mg280) II	2.9 A	
MT9455	tbh-1(n3247) X	2.9 A	
MT15620	cat-2(n4547) II	2.9 B	
IV111	cat-2(e1112) II; ueEx51 [p27::cat-2-sl2-GFP; Pelt-2::GFP]	2.9 C	
IV552	cat-2(e1112) II; ueEx355 [Pdat-1p19::cat-2-sl2-GFP; Pelt-2::GFP]	2.9 C	
LX645	dop-1(vs100) X	2.11	
LX702	dop-2(vs105) V	2.11	
LX703	dop-3(vs106) X	2.11	
RB1254	C52B11.3(ok1321) X	2.11	
LX705	dop-1(vs100) dop-3(vs106) X	2.12 A	

Table 2.1: List of strains used in Chapter 2, continued

Strain Name	Genotype	Figure	Notes
LX706	dop-2(vs105) V; dop-1(vs100) X	2.12 A	
IV984	dop-4(ok1321) dop-1(vs100) X	2.12 A	Created for this study
IV985	dop-2(vs105) V; dop-4(ok1321) X	2.12 A	Created for this study
IV986	dop-4(ok1321) dop-3(vs106) X	2.12 A	Created for this study
LX734	dop-2(vs105) V; dop-1(vs100) dop-3(vs106) X.	2.12 B	
CF2805	dop-2(vs105) V; dop-4(ok1321) dop-1(vs100) dop-3(vs106) X	2.12 B	

7. References

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Chapter 3:

Mechanosensation and serotonin

1. Introduction

This chapter will cover additional data that start to probe additional mechanisms behind *C. elegans* behavior in response to a predator. Biogenic amine signaling is a common motif in fear learning in invertebrates and can involve dopamine or serotonin, as summarized in Chapter 1. Dopaminergic signaling appears to coordinate the motor element of the learned fear response in *C. elegans*, downstream of fear acquisition. To investigate the upstream pathway, I analyzed the role of mechanosensation in this behavior, as the requirement for a biting predator suggests that mechanosensation of the bite is the unconditioned stimulus responsible for provoking the fear response. Additionally, I investigate the role of serotonin in this behavior, as a candidate screen in Chapter 2 identified serotonin as well as dopamine as required for the egg distribution behavior.

Mechanical stimuli to the *C. elegans* body can signal different things and elicit different types of behavior depending on its location and its severity. Touch-responsive receptors have been identified using gentle touch (usually a stroke from an eyelash or eyebrow hair) or harsh touch (a prod from a wire) [1], [2]. Gentle touch to the body, avoiding the head, tail, or vulva, evokes movement away from the touch. These types of touches are sensed by six touch responsive neurons (ALM, PLM, AVM, and PVM) [3]. Harsh touch can be sensed by animals that do not respond to gentle touch and is mediated by PVD neurons [2]. Tap, produced by either tapping or dropping the agar dish, activates both anterior and

posterior touch responsive neurons and generally evokes reversals [4], [5]. However, none of these types of touch are necessarily similar to the bite of a predator. Since predator biting is required for *C. elegans* to change its behavior, I wondered which of the identified touch sensors could also be bite sensors.

Mechanosensation and dopamine have already been linked in previous studies. In fact, dopamine-releasing neurons are also mechanosensory neurons [6]. Additionally, activation of body touch neurons can signal to and activate dopamine neurons [7]. Dopamine also regulates response to touch over time. For example, dopamine reduces habituation to tap response, which allows worms on food to retain their sensitivity and response to tap [8]. Additionally, dopamine is required for *C. elegans* to prefer denser patterns of PDMS pillars [9]. Given these roles of dopamine in sensitizing aversive responses as well as monitoring and choosing environments based on touch, identifying the role of mechanosensation in predator-evoked behaviors could help in determining the mechanisms behind which those behaviors arise.

In the previous chapter, I demonstrated that bioamine signaling was required for the off-lawn egg-laying behavior induced by predator exposure. In addition to the *cat-2*, a *tph-1* mutant was shown to be defective in response to predator. *tph-1* encodes tyramine hydroxylase which catalyzes the rate-limiting step in serotonin synthesis [10]. Serotonin is involved in learning in *Aplysia*, where it enforces long-term memory by inducing CREB-1 phosphorylation [11]. Serotonin and dopamine also often act together to modulate the same behavior. For example, while dopamine signaling is required for basal slowing when encountering a lawn of food, serotonin can enhance the slowing response if the animal is starved [6]. In this way, dopamine modulates the basal behavior while serotonin modulates it in an experience-dependent manner. Therefore, serotonin is a possible neurotransmitter that

could act upstream of dopamine in this fear learning assay, and could be required for acquisition of the memory of predator exposure.

2. Mechanosensation

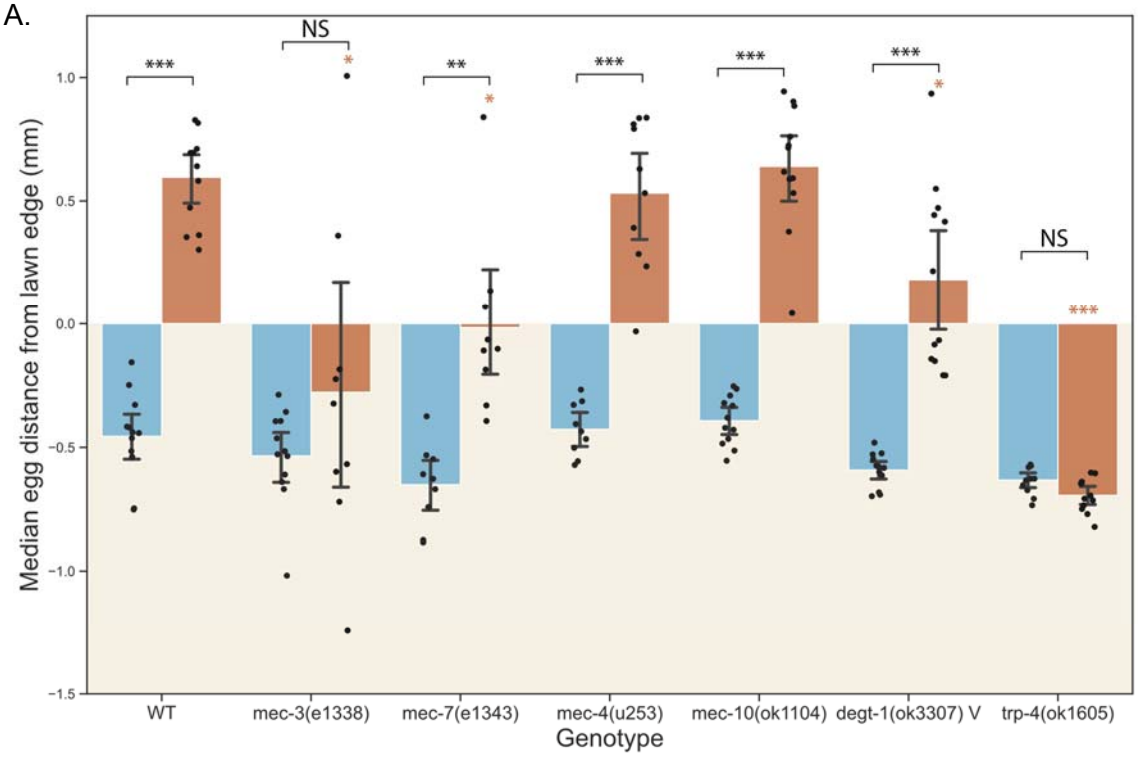
I tested an array of mechanosensation defective mutants to determine whether we could find either a bite sensor or a way that *C. elegans* senses the environment (Figure 3.1 A). Two mutants, *mec-3*(*e1338*) and *mec-7*(*e1343*), lack functional TRNs, with the *mec-3* mutant also lacking functional PVD neurons, a type of neuron with dendrites along the entire length of the body that mediates harsh touch responses [12], [13]. Both *mec-3* and *mec-7* mutants showed behavioral defect in responding to predator, with *mec-3* showing the stronger defect, indicating the importance of both PVD and TRNs for conducting this behavior. Mutations in the DEG/ENaC proteins *mec-4* and the *mec-10*, which are important for gentle touch but retain sensitivity to harsh touch [14], [15], did not affect the behavior. The mutant in *degt-1*, which sense harsh touch in PVD [16], [17], can still change its behavior in a predator-dependent way but is slightly defective. DEGT-1 has been reported to co-localize with MEC-10 and therefore may be part of the same type of channel [17], so the lack of defect in the mec-10 mutants suggest that *degt-1* may have an additional role to play in sensing bites that is independent of its known function with *mec-10*. However, the *degt-1* mutation failed to completely abolish the phenotype, so it may a single channel of multiple that are capable of sensing predator bites. It is unsurprising that a single touch-defective mutant would not abolish the behavior completely, as mechanosensation is an extremely important sense for the worm to use to escape predators and likely is sensed redundantly by multiple genes. Additionally, this strain carrying *degt-1*(*ok3307*) has recently been shown to carry a linked mutation, rpm-1(ju1928), which causes overextension of the PLM axon [18].

Re-testing this phenotype using the outcrossed strain with the *rpm-1* mutation removed should clarify whether the *degt-1* mutation is responsible for the slightly defective behavior.

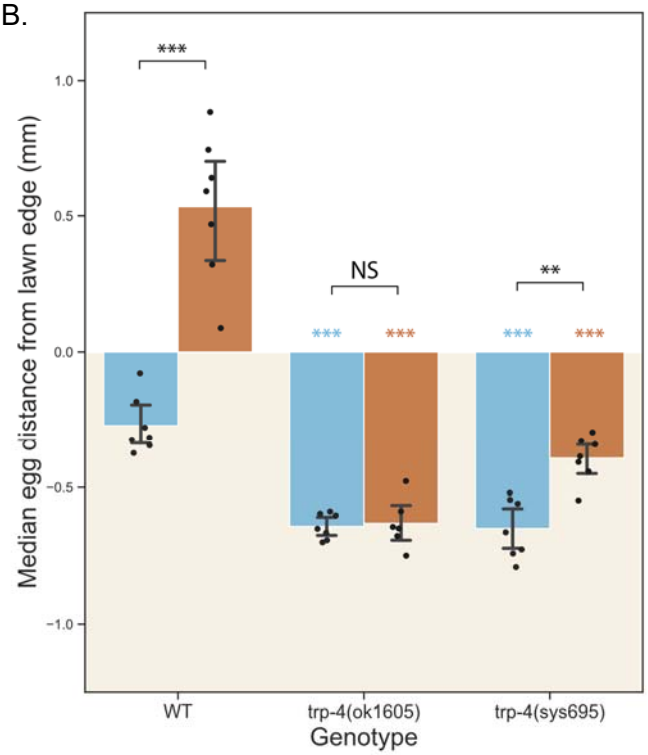
A *trp-4* allele *ok1605* is severely defective in the behavior, with no change between predator-exposed and control conditions. A second *trp-4* allele *sys695* also displays strongly defective behavior, although there is a difference between control and predator conditions (Figure 3.1 B). Both alleles are deletions that remove large portions of the coding region. The *sys695* allele removes the transmembrane domain [19], and the *ok1605* allele removes exons 12-14. TRP-4 is a pore-forming TRPN channel [20] that expresses in CEP neurons to detect the pressure exerted by bacteria [21]. C. elegans can distinguish between and show preference for different textures in a TRP-4-dependent manner [9]. Both *trp-4* alleles also demonstrate a basal difference in egg-laying distribution than wildtype; they tend to lay eggs closer to the lawn's center. This suggests that TRP-4 affects basal egg-laying position relative to food, which is unsurprising given the role of TRP-4 in sensing bacterial presence. TRP-4 also contributes to posterior harsh touch response in PVD [22]. Therefore, it is possible that TRP-4's role in PVD sensing rather than bacteria sensing leads to the change in egg distribution as a potential bite sensor.

Figure 3.1 Mechanosensory neurons are required for behavior. **A.** A candidate screen of various genes related to mechanosensation. **B.** Two alleles of *trp-4* are defective in egg distribution behavior. Error bars represent 95% confidence interval. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between N2/WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant differences between pairwise comparisons are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

A.


Median egg distance from lawn edge (mm)
1.0
0.5
0.0
−0.5
−1.0
−1.5
***
NS
*
**
*
***
*
***
*
NS
***
WT
mec-3(e1338)
mec-7(e1343)
mec-4(u253)
mec-10(ok1104)
degt-1(ok3307) V
trp-4(ok1605)
Genotype

B.


Median egg distance from lawn edge (mm)
1.0
0.5
0.0
−0.5
−1.0
***
NS
**
***
***
***
***
WT
trp-4(ok1605)
trp-4(sys695)
Genotype

3. Serotonin

While screening mutants that affect biogenic amine signaling, both mutations that affect dopamine and serotonin signaling decreased response to predator. A single-copy mosSCI insertion of *tph-1* using its endogenous promoter [23] was able to rescue the behavior. Serotonin is synthesized in ADF, NSM, and HSN [10]. However, technical problems with transgenic strains prevent testing transgenic rescues of *tph-1* in these different neuronal types, so it is unknown which neurons need to synthesize serotonin to modulate response to predator.

At least five serotonin receptors are confirmed in *C. elegans* – SER-1, SER-4, SER-5, SER-7, and MOD-1 [24]–[29]. Additionally, MOD-5 is a serotonin reuptake transporter [30]. A screen of single mutants in the known serotonin receptors and MOD-5 revealed a potential candidate receptor, SER-7. The *ser-7* allele *tm1325* removes the fifth exon of the gene [31]. However, a CRISPR allele *vq2* removing the entire *ser-7* gene [32] failed to recapitulate the phenotype after backcrossing. Attempts to rescue *ser-7* transgenically by injecting the full gene, including its endogenous promoter, failed to produce any progeny carrying the transgene. This is consistent with the report in [31] where the authors stated that extremely low concentrations of transgene were needed and that higher concentrations were toxic. The inconsistency between the phenotypes of the two alleles could be that the *tm1325* is not a null allele, or there are additional linked mutations in the strains that are contributing the phenotype. SER-7 responds to serotonin by increasing pharyngeal pumping rate [31]. SER-7 also plays a role in learning by responding to increased serotonin release by ADF on familiar food to increase pharyngeal pumping rate [33]. Whether SER-7 is involved through modulating pharyngeal pumping rate or indeed if SER-7 is involved at all is still unclear due to the differing results from the two alleles. Even if SER-7 is not the downstream receptor

that responds to serotonin in predator exposure, there are probably additional, yet uncharacterized serotonin receptors [26] which may also be involved in this behavior.

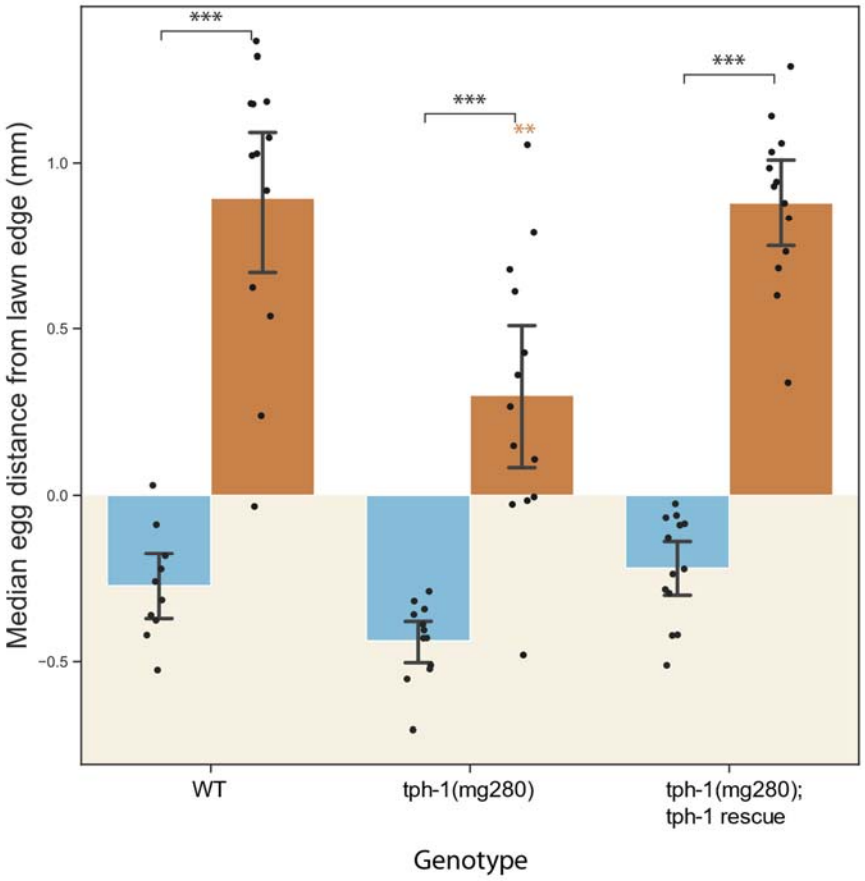
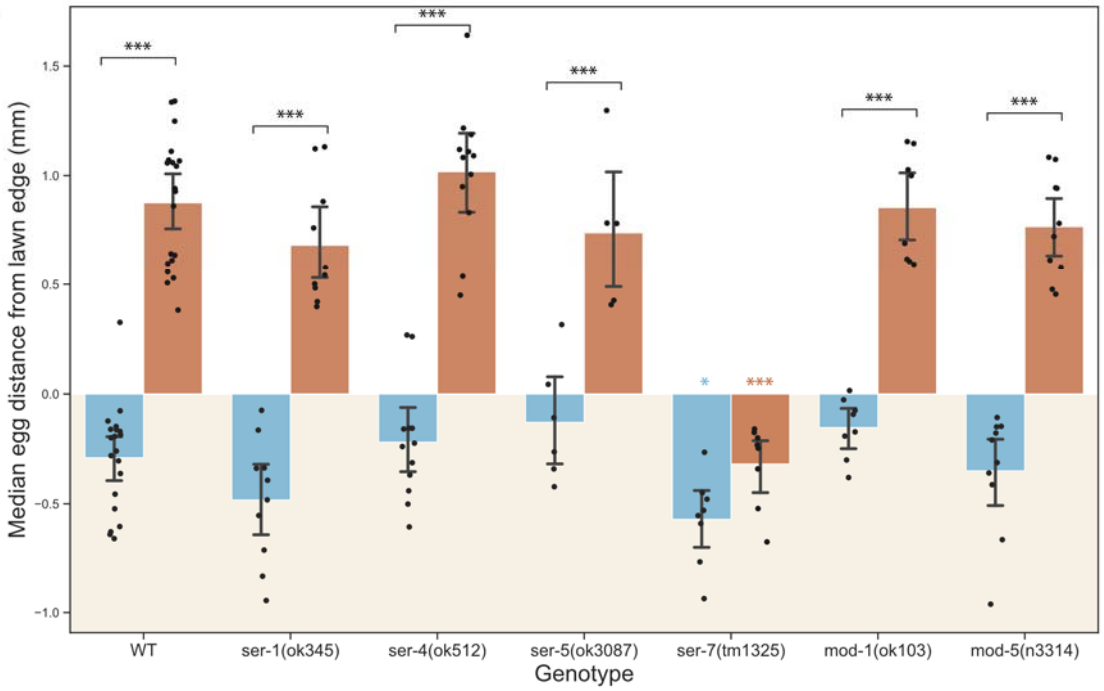


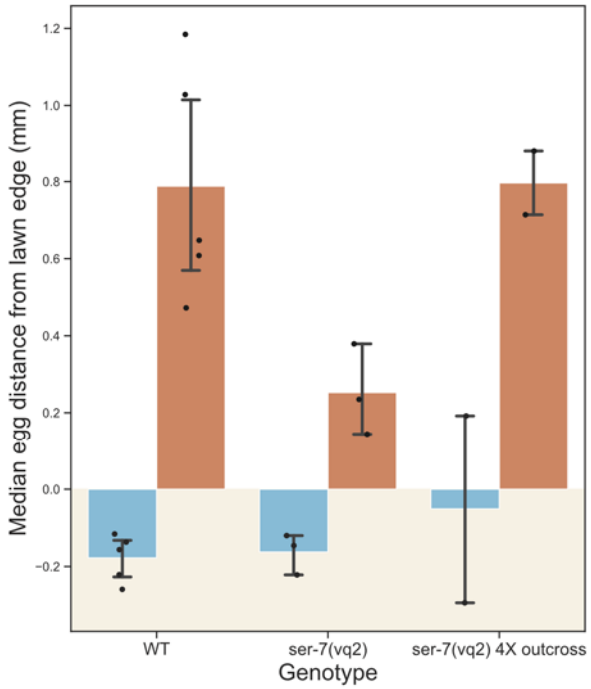
Figure 3.2 A *tph-1* mutant is defective in predator-responsive behavior, but a genomic rescue of *tph-1* rescues behavior. Error bars represent 95% confidence interval. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between N2/WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant differences between pairwise comparisons are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$.

Figure 3.3 It is unclear which serotonin receptors may be involved in the egg distribution behavior. **A.** A candidate screen of all known serotonin receptors and the serotonin reuptake transporter. Error bars represent 95% confidence interval. Pairwise comparisons are performed between predator/control conditions within the same strain (black asterisks), as well as between N2/WT and mutants of the same predator/control condition (blue asterisk = comparison between WT/control and mutant/control; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). Significant differences between pairwise comparisons are tested with Welch's t-tests. P-values adjusted with Holm-Bonferroni correction. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$, NS=$p>0.05$. **B.** An outcrossed CRISPR allele deleting the entire *ser-7* gene loses its defective phenotype. No statistics are shown for this panel due to low sample number.

a.


Median egg distance from lawn edge (mm)
1.5
1.0
0.5
0.0
−0.5
−1.0
***
***
***
***
***
***
*
***
WT
ser-1(ok345)
ser-4(ok512)
ser-5(ok3087)
ser-7(tm1325)
mod-1(ok103)
mod-5(n3314)
Genotype

b.


Median egg distance from lawn edge (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
WT
ser-7(vq2)
ser-7(vq2) 4X outcross
Genotype

4. Methods

Assays for this chapter are conducted the same as in Chapter 2.

Table 3.1 List of strains used in Chapter 3

Strain	Genotype	Figure	Notes
N2	Wild-type	3.1, 3.2, 3.3	From CGC
CB1338	mec-3(e1338) IV	3.1 A	
CB1477	mec-7(e1343) X	3.1 A	
TU253	mec-4(u253) X	3.1 A	
RB1115	mec-10(ok1104) X	3.1 A	
VC2633	rpm-1(ju1928) degt-1(ok3307) V	3.1 A	Carries linked mutation in rpm-1
GN716	trp-4(ok1605) I	3.1	Outcrossed 4X; from M. Goodman
TQ296	trp-4(sy695) I	3.1 B	
MT15434	tph-1(mg280) II	3.2	
CX13228	tph-1(mg280) II; kySi56 [loxP-tph-1(genomic)] IV	3.2	From C. Bargmann
DA1814	ser-1(ok345) X	3.3 A	
AQ866	ser-4(ok512) III	3.3 A	
RB2277	ser-5(ok3087) I	3.3 A	
DA2100	ser-7(tm1325) X	3.3 A	
MT9668	mod-1(ok103) V	3.3 A	
MT9772	mod-5(n3314) I	3.3 A	
VV207	ser-7(vq2) X	3.3 B	From M. Petrascheck
IV901	ser-7(vq2) X	3.3 B	Outcrossed 4X; created for this study

5. Discussion and future directions

The screen of known mechanosensory genes confirmed that mechanosensory neurons are required for behavior, which is expected due to the requirement of a predator that can bite. The screen also revealed that *degt-1* may be involved in this behavioral paradigm, and *trp-4* probably is. The *degt-1* phenotype was incompletely defective, but its known role for harsh touch sensation makes it a good candidate for a bite sensor. It is

possible that a double mutant in *degt-1* and *mec-10*, due to their role together in a mechanosensory complex [34], could completely abolish response to predator. However, the *mec-10* single mutant behavior was indistinguishable from wild-type. Therefore, DEGT-1 may co-localize with some other unknown channel.

Further studies will need to be conducted to determine whether TRP-4 acts to sense information about bacteria location or whether TRP-4 acts in the bite-sensing circuit. If TRP-4 expression in CEP restores behavior, then perhaps the main role of TRP-4 is in sensing bacteria, which results in dopamine release. However, if TRP-4 expressed in PVD rescues the behavior, then TRP-4 may be responsible for bite sensing via PVD. Since the predator bite can occur throughout the body, and PVD's dendrites cover the majority of the worm's body, PVD could be a predator bite-sensing neuron. It is also possible that TRP-4 rescue requires expression in all dopaminergic neurons, where it is natively expressed [19], [35]. In this case, it may be a direct mechanosensory sensor in these neurons, but it also may be sensing and responding to other cues. TRP channels in other organisms including mammals respond to a variety of stimuli. They can also respond downstream from other pathways rather than responding specifically to mechanosensation [36]. TRP-4 in *C. elegans* has been shown to respond directly to touch to evoke MRCs in CEPs [21], and to be required to calcium increase in CEPs upon food entry [37] but its specific role in other dopaminergic neurons has yet to be explored.

Additionally, it would be interesting to see when serotonin is necessary in the assay. Dopamine was required to tune the execution of the behavior, but not to acquire the memory. Perhaps serotonin is a signal that establishes memory and is therefore required in the beginning of the assay rather than the end. To answer this question, exogenous serotonin could be used to pre-treat serotonin-defective *C. elegans* to see if this rescues the behavior.

Serotonin should not be used during the assay itself when the predators are present on the plate, as serotonin activates predatory behavior in *Pristionchus* [38].

To further investigate which serotonin receptors might be important in this behavior, double mutants and possibly triple mutants should be investigated in case multiple receptors work together, as seen in the dopamine receptor results. The question of whether ser-7(tm1325) actually causes defective behavior, or if there is an unknown linked mutation, could be addressed by rescuing *ser-7* in the *tm1325* background. Because of the difficulty with transgenic rescue, single copy insertion techniques like mosSCI could be utilized. Deletion of exon five using CRISPR could also confirm if the same deletion found in *tm1325* affects the predator-induced behavior.

Overall, further investigation into the roles of mechanosensation and serotonin in this predator-induced behavior paradigm could illuminate the mechanism of learning this behavior. Determining the upstream circuit should start at mechanosensation with a bite sensor, and serotonin could be the neurotransmitter that is required to integrate information about the memory of biting.

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Chapter 4:

Conclusions

1. Predator-evoked behavior in *C. elegans*

Antipredator behaviors vary by prey and predator species. General strategies can include increased vigilance, hiding, or attempting to deter the predator through posturing. To measure the degree of responsiveness to a predator, first we must find out what behavior to expect from the prey. What then, does *C. elegans* do when it learns of the presence of a nematode predator like *P. uniformis*?

Both species in this case cannot see, so their ability to sense at a distance is limited to olfaction or chemosensation. Some strains of *P. pacificus* secrete sulfolipids that induce aversion in *C. elegans* [1]. We proposed that these sulfolipids secretions are a kairomone that benefits *C. elegans* by warning them of the potential presence of a predator. However, evolutionary pressure to lose the kairomone could explain why some strains of *P. pacificus* in our previous study had less aversive secretions. While chemosensation of predator-released compounds can contribute to prey avoidance of predators, it did not play a role in the egg distribution behavior discussed in Chapters 2 and 3 as shown by the lack of avoidance of *P. uniformis*-conditioned media.

C. elegans can learn about the presence of predators through mechanosensation. If the *C. elegans* feels a bite, the threat is clearly at hand. *C. elegans* can already use mechanosensation to avoid traps set by predatory fungi [2], but fungi are stationary while

Pristionchus predators roam around the environment. Adult *C. elegans* can easily evade bites compared to larvae [3], so single bite sensations themselves are not particularly dangerous to the adult. Unlike chemosensation of a kairomone that may not be present in all predators, feeling a bite is definitive proof of a predator.

In the egg distribution assay, it takes around six hours of experiencing bites for *C. elegans* to change their behavior. The presence of a non-biting predator did not induce any behavioral change even after twenty hours. It is possible that the number of bites received during that time could shift the behavior change – that is, there may be a threshold of events needed before *C. elegans* decides to change its behavior. However, even using the *P. pacificus* strain RS5194 with a high bite probability [4], a strong phenotype was not seen before six hours. Therefore, experiencing these bites over a long amount of time is important for behavioral change.

After a period of enough predator exposure, *C. elegans* changes how it interacts with its food environment. When removed from the training plate with predator and placed in a new arena, egg distribution on even food is not changed between predator-exposed and control animals. However, egg distribution changes in arenas with a small lawn and arenas with a smeared lawn. Predator-exposed animals prefer the edge of the lawn and leave the lawn more frequently in the small lawn arena while predator-exposed animals lay eggs with high frequency in the smeared lawn, basically matching the behavior of animals in an arena covered evenly in food (Figure 4.1). This behavior suggests that predator-exposure alters foraging decisions in *C. elegans*. *C. elegans* can sample the environment and choose the best source of food – that is, a source of food that sustains the highest level of growth [5]. However, predator exposure alters this preference so that rather than choose the dense bacterial lawn in the center, *C. elegans* chooses highly variable areas of food.

Altered foraging behavior is a common response to predator presence. In large-scale example, reintroduction of wolves into Yellowstone National Park affected the foraging behavior of their prey, elk. Female elk with calves in wolf territory spent less time grazing and more time performing vigilance behaviors [6]. In another example where a rat is confronted with a moving robotic predator, the rats in the presence of this predator take longer to acquire the same amount of food because their hesitancy to approach [7], [8]. The choice to inhabit areas of high variability could be beneficial if *C. elegans* predators also prefer to inhabit areas with dense food. The areas of high variability could reduce the interaction with predators while still providing some food.

In summary, *C. elegans* detects predator presence primarily through mechanosensation. This mode is probably a more reliable way to learn about new threats than relying on sensing predators through secreted chemicals. After a period of learning, *C. elegans* switches foraging strategy to move away from dense food areas to high variability areas that still have some food. This strategy probably decreases their consumption of high-quality food but balances their need to eat with avoiding predators.

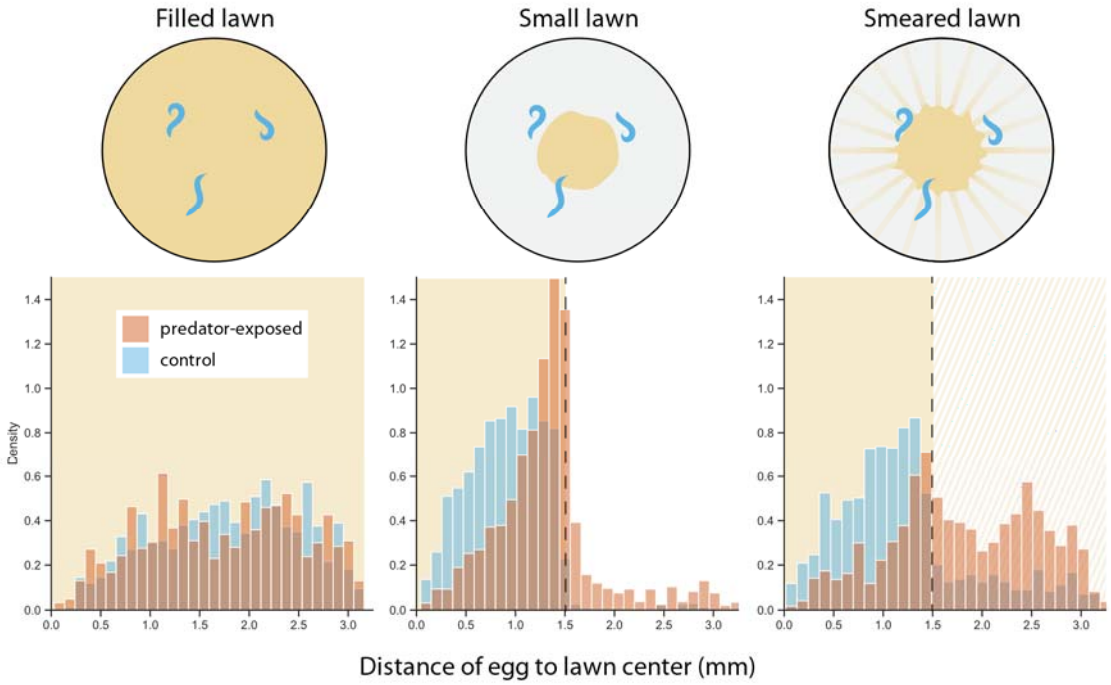

Figure 4.1 Predator exposure alters foraging strategy. Pooled egg distributions across timepoints from data from Figure 2.14 to illustrate egg location preference relative to the lawn. Bacteria indicated on graph by beige color blocks, with striped block indicating smeared bacteria. Dashed line indicates average lawn edge. Y-axis is density, with independent density normalization.

2. Molecular mechanisms

We identified biogenic amine signaling as necessary for the shifted egg distribution behavior. Both serotonin and dopamine are required, as well as the vesicular monoamine transporter (Figure 2.9 A). In Chapter 2, we discuss the role of dopamine in this behavior.

Decreased dopamine in the *cat-2(e1112)* mutant resulted in depressed off-lawn movement, while increased dopamine in the *dat-1(ok1605)* mutant resulted in increased off-lawn movement (Figure 2.10). The overall location traces of both control and shifted up in the *dat-1* mutant, suggesting that excess dopamine itself can alter foraging strategy. To test this hypothesis, I added exogenous dopamine to the test arena and transferred *cat-2* mutants after training with or without a predator. Exogenous dopamine rescued *cat-2* mutant behavior in predator-exposed animals essentially back to wildtype levels, and had a lesser effect on *cat-2* mutants that were not exposed to predator (Figure 2.15). This confirmed that dopamine signaling primarily affects the execution of the altered foraging strategy.

Downstream of dopamine release, we identified *dop-1*, *dop-2*, and *dop-3* as important for this behavior as single and double mutants had weaker phenotypes than the triple mutant. However, the quadruple mutant with an additional *dop-4* mutation had a similar phenotype to the triple mutant, indicating that *dop-4* is dispensable (Figure 2.12). DOP-1 and DOP-4 are D1-like receptors, and DOP-2 and DOP-3 are D2-like receptors. Activation of D1-like receptors leads to increased cyclic AMP, while D2-like receptor activation inhibit adenylyl cyclase [9]–[11]. In this way, the actions of the two types of receptors often antagonize each other. Indeed, DOP-1 and DOP-3 have been shown to antagonize each other in basal slowing behavior, paralysis induced by exogenous dopamine, and crossing an aversive copper barrier [12], [13]. However, the results from the egg distribution behavior suggest that DOP-1, DOP-2, and DOP-3 can synergize.

Dopamine controls aspects of *C. elegans* foraging including slowing down when encountering food [14] and adjusting search patterns when removed from food [15], [16]. We propose a model where bites from predators alter the dopaminergic circuit to respond to dopamine differently. This could be done in a few different ways. One possibility is that tonic dopamine levels increase in predator-exposed animals due to prolonged biting. If basal dopamine is already high, food-associated dopamine release may need to be higher to stand out from background. The high contrast of the areas with bacterial variability can result in more dopamine release [16], so they become preferred over the constant, though denser, environment. *cat-2* mutants do not require dopamine during the training period to respond to exogenous dopamine. Therefore, other changes like dopamine receptor expression may be altered during the training period to affect dopamine sensitivity. Dopamine receptor expression decrease has been seen in human patients with chronic drug use [17]. It is also possible that rather than decreasing overall receptor expression, again so that higher contrast is favored.

Decreased serotonin in the *tph-1(mg280)* mutant also decreased predator responsiveness. This was rescued with a single-copy insertion (Figure 3.2), but it was inconclusive which serotonin receptor might be responsible for downstream signaling. Serotonin has been shown to modulate dopamine-dependent behaviors by coding internal state. For example, *C. elegans* normally slows down when entering bacteria, and this slowing response is dependent on dopamine released after sensing the differential texture. When *C. elegans* are starved, they slow down even more in a behavior called enhanced slowing. In the case of enhanced slowing, dopamine modulates the food-related touch inputs while serotonin contributes information about internal state [14]. It is possible in this egg distribution paradigm that serotonin can code the internal state of predator exposure while dopamine allows the animal to tune its environmental preference.

3. Unanswered questions

As mentioned above, the mechanism by which predator training alters *C. elegans'* response to dopamine is yet to be elucidated. The proposed models of either tonic dopamine elevation or altered dopamine receptor expression could be tested by imaging dopaminergic neurons at different points during the assay or by conducting qPCR experiments of the dopamine receptors of interest.

The involvement of serotonin in this behavior is still mostly unknown. Serotonin is synthesized in ADF, NSM, and HSN [18]. Cell-specific rescue could suggest which aspect of the behavior serotonin controls. NGM is involved in sensing food ingestion [19] and modulating feeding behavior through pumping rate [20] while HSN controls egg-laying [21]. ADF also regulates pumping rate [22] and aversive responses [23]. Rescue of behavior by restoring serotonin synthesis to one of these neurons could suggest whether serotonin is needed for sensing food, for regulating egg-laying, or for regulating locomotion. Serotonin can also be released by other neurons by re-uptake through MOD-5 [24], but a *mod-5* mutant did not display any behavior defect so it is likely not involved.

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