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## What can a worm learn in a bacteria-rich habitat?

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### Summary

With a nervous system that has about a billion times fewer neurons than the human brain, *C. elegans* was initially not regarded as a model for studies on learning. However, the collective effort of the *C. elegans* field in the past several decades has shown that the worm displays plasticity in its behavioral response to a wide range of sensory cues in the environment. As a bacteria-feeding worm, *C. elegans* is highly adaptive to the bacteria enriched in its habitat, especially those that are pathogenic and pose a threat to survival. It uses several common forms of behavioral plasticity that last for different amounts of time, including imprinting and adult-stage associative learning, to modulate its interactions with pathogenic bacteria. Probing the molecular, cellular and circuit mechanisms underlying these forms of experience-dependent plasticity has identified signaling pathways and regulatory insights that are conserved in more complex animals.

### *C. elegans* senses and responds to diverse environmental cues

Animals live in different ecological niches that are characteristic of different chemical, physical and biological cues and have likely evolved sensorimotor systems that are able to detect and respond to the environmental conditions of their habitats. *C. elegans* feeds on bacteria and is often found in decaying fruits or other organic matters that are rich in bacteria (Felix and Duveau, 2012; Frezal and Felix, 2015; Samuel et al., 2016). It navigates its environment by detecting and responding to various chemical cues, including odorants and salts, temperature, pheromones, gases, as well as mechanical stimuli [(Figure 1) and (Aoki and Mori, 2015; Bargmann, 2006; Brandt et al., 2012; Bretscher et al., 2008; Butcher et al., 2007; Chalfie, 2009; Cheung et al., 2005; de Bono and Maricq, 2005; Goodman et al., 2014; Goodman and Sengupta, 2019; Gray et al., 2004; Hallem et al., 2011; Hao et al., 2018; Jeong et al., 2005; Kaplan and Horvitz, 1993; Kim et al., 2009; Macosko et al., 2009; Pierce-Shimomura et al., 2001; Reddy et al., 2011; Schafer, 2015; Srinivasan et al., 2008; Srinivasan et al., 2012; White and Jorgensen, 2012; White et al., 2007)]. Some of the odorants that are attractive to *C. elegans* can be produced by plants and may serve as cues representing an environment that is abundant in bacteria. In addition, *C. elegans* is known to navigate within a thermal gradient and the ambient temperature significantly regulates the development and life span of a worm. The sensorimotor response to chemical cues and temperature have been extensively studied in *C. elegans*. For example, a few ciliated sensory neurons use G proteins, cyclic nucleotide-gated channels (CNGs), and G-protein coupled

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seven-transmembrane receptors to detect odorants. The calcium-permeable CNGs transform odorant information into intracellular signals, which produce intercellular signals to engage postsynaptic interneurons and downstream motor neurons to generate movement towards or away from the odorants (Bargmann, 2006; de Bono and Maricq, 2005). Similarly, the major sensory neurons, as well as their intracellular signaling pathways, that perceive and respond to external salt concentration, ambient temperature gradient, pheromones, gases and mechanical cues have been identified and characterized (Aoki and Mori, 2015; Bargmann, 2006; Brandt et al., 2012; Bretscher et al., 2008; Butcher et al., 2007; Chalfie, 2009; Cheung et al., 2005; de Bono and Maricq, 2005; Goodman et al., 2014; Goodman and Sengupta, 2019; Gray et al., 2004; Hallem et al., 2011; Hao et al., 2018; Jeong et al., 2005; Kaplan and Horvitz, 1993; Kim et al., 2009; Macosko et al., 2009; Pierce-Shimomura et al., 2001; Reddy et al., 2011; Schafer, 2015; Srinivasan et al., 2008; Srinivasan et al., 2012; White and Jorgensen, 2012; White et al., 2007). The behavioral strategies and the underlying neural circuits through which *C. elegans* navigates in a sensory environment are also intensively investigated, such as those described in (Aprison and Ruvinsky, 2019; Bargmann, 2006; Chalasani et al., 2007; de Bono and Maricq, 2005; Donnelly et al., 2013; Goodman and Sengupta, 2019; Gordus et al., 2015; Gray et al., 2005; Iino and Yoshida, 2009; Ikeda et al., 2020; Jang et al., 2012; Kaplan et al., 2020; Kato et al., 2015; Kunitomo et al., 2013; Li et al., 2014; Liu et al., 2018; Luo et al., 2014; Macosko et al., 2009; Pierce-Shimomura et al., 1999; Schafer, 2015; Tsalik and Hobert, 2003; Venkatachalam et al., 2016; Wen et al., 2018; White et al., 2007). The molecular, cellular and circuit bases for these sensorimotor responses provide the substrates for experience-dependent regulation to generate learning. The studies that investigate various forms of learning in *C. elegans* have been reviewed elsewhere (Alcedo and Zhang, 2013; de Bono and Maricq, 2005; McDiarmid et al., 2019; Sasakura and Mori, 2013). Here, we will focus on several learning paradigms that regulate the interactions between *C. elegans* and pathogenic bacteria.

## Environmental cues induce plasticity across different timescales

*C. elegans* displays both adaptation and habituation, two common forms of non-associative learning (Figure 1). *C. elegans* is attracted to several chemical odorants, such as Isoamyl alcohol and benzaldehyde; however, prolonged exposure to these volatile chemicals reduces the sensory response to the odorants and generates adaptation that lasts for a couple hours (Colbert and Bargmann, 1995; Inoue et al., 2013; Kaye et al., 2009). It is shown that during adaptation the endogenous small RNA (endo-siRNA)-mediated gene expression regulation in the sensory neuron that detects isoamyl alcohol and benzaldehyde downregulates a guanylyl cyclase that is critical for the G-protein coupled signaling pathway underlying the sensing of the odorants (Juang et al., 2013). These results reveal a novel function of endo-siRNA pathways in regulating gene expression in response to olfactory experience. In addition, the worm reverses from a mild mechanical stimulus that is delivered to its body or nose and senses the stimulus using receptor neurons, several of which contain distinct morphological features (Chalfie, 2009; Kaplan and Horvitz, 1993; Schafer, 2015). Tapping the cultivating plate also generates mechanical stimuli that trigger reversals. However, tapping for multiple times reduces the amplitude of the reversals (Rankin et al., 1990). This type of behavioral changes is analogous to habituation previously characterized in *Alysia*

and cats, where multiple stimulation with a benign mechanical stimulus leads to a reduction in response (Bailey and Chen, 1983; Spencer et al., 1966). Repeated habituation training under certain conditions can generate memory that lasts for 24 hours (Rose et al., 2002).

In addition to non-associative learning, previous studies have shown that olfactory responses can be respectively enhanced or weakened by pairing odorant exposure with the presence or absence of food, which presumably represents an appetitive or aversive environment (Figure 1). Various neuronal circuits and molecular pathways have been characterized in regulating these associative learning behaviors [(Alcedo and Zhang, 2013; de Bono and Maricq, 2005) and the references therein]. *C. elegans* also remembers the salt concentration under its cultivation condition and seeks this concentration when tested in a salt gradient after the training. However, if the worm is kept at a salt concentration in the absence of food, it avoids the concentration during the post-training rest (Kunitomo et al., 2013; Luo et al., 2014; Saeki et al., 2001; Tomioka et al., 2006). As a critical condition, the cultivation temperature significantly modulates the navigation of the worm in a temperature gradient during post-training tests (Aoki and Mori, 2015; Biron et al., 2006; Goodman et al., 2014; Goodman and Sengupta, 2019; Hedgecock and Russell, 1975; Mori and Ohshima, 1995). Some of these forms of behavioral plasticity resemble associative learning identified in vertebrate animals and in fruit flies. While a one-time massed training in these paradigms often generates memories for a couple hours, spaced training can generate long-term memories that last for 16 hours (Kauffman et al., 2010).

## Experimental power of *C. elegans* facilitates dissection of plasticity mechanisms

The ease of applying forward and reverse genetic approaches to characterize gene function in *C. elegans* and the knowledge of genetic identities and synaptic connectivities of the worm neurons facilitates studies on learning and behavioral plasticity in *C. elegans* in several important ways:

1. Because the worm neurons are defined in their genetic making and synaptic connectivity, we are able to identify the neurons where the gene products implicated in learning are generated and act, as well as their presynaptic and post-synaptic neurons. These analyses provide us with knowledge on neuronal circuits underlying various forms of learning behaviors.
2. By applying *in vivo* imaging and genetic manipulations, we can identify experience-dependent changes in the activity and the connectivity of the learning circuit that are correlated with behavioral changes and characterize the causality of these changes in generating learned behavior.
3. Once the key neurons underlying learning are identified, we can also analyze gene expression in these neurons in naive and trained animals in order to identify genes that display training-correlated changes in their expression and address the function of these molecules in learning.

4. Meanwhile, the ease of performing genetic analyses in the *C. elegans* nervous system also makes it feasible to conduct genetic screens in order to identify new functions of characterized genes and pathways in learning, as well as identify new genes with previously unknown functions.

## Interactions with pathogenic bacteria that modulate behavior

*C. elegans* feeds on bacteria in the wild and in the laboratory setting. A wide range of different bacteria strains, including many in the *Pseudomonas* genus, are found to be associated with *C. elegans* isolated from its natural habitats (Felix and Duveau, 2012; Frezal and Felix, 2015; Samuel et al., 2016; Schulenburg and Felix, 2017). While some of these bacteria serve as food sources, others are pathogenic and kill *C. elegans* through infections or with secreted toxins [(Hoffman and Aballay, 2019; Irazoqui et al., 2010; Kim and Ewbank, 2018) and the references therein]. Because bacteria play a vital role in the development and survival of *C. elegans*, it is conceivable that *C. elegans* has evolved diverse strategies to mediate its interactions with the environmental bacteria.

Bacteria produce multiple types of sensory cues that can be used by the worm to detect and respond to the microbes. In addition to odorants, bacteria also produce water-soluble metabolites, generate or alter concentration of gases. The border and the texture of a bacterial lawn may also generate mechanical stimulation to moving worms. These bacteria-derived sensory cues act in a combinatorial manner to elicit behavioral responses in *C. elegans* [(Bargmann et al., 1993; Brandt and Ringstad, 2015; Bretscher et al., 2008; Calhoun et al., 2015; Cheung et al., 2005; Flavell et al., 2013; Gramstrup Petersen et al., 2013; Gray et al., 2004; Ha et al., 2010; Hallem et al., 2011; Hao et al., 2018; Harris et al., 2019; Meisel et al., 2014; Ooi and Prahlad, 2017a; Pradel et al., 2007; Reddy et al., 2011; Rhoades et al., 2019; Sawin et al., 2000; Tran et al., 2017), Flavell and Kim this issue and the references therein]. The diversity of the sensory cues is consistent with multiple signaling pathways that are identified to mediate bacteria-worm interactions.

### Adult-stage learning of pathogenic bacteria

Some pathogenic bacteria, such as the *Pseudomonas aeruginosa* strain PA14, infect *C. elegans* after being ingested, which leads to a slow death of the worm during a course of several days (Tan et al., 1999). Thus, pathogenic bacteria likely signal both food and danger to the worms. The odorants of several pathogenic bacteria, including PA14, are attractive to the worms that are cultivated under the standard condition by feeding on *E. coli* OP50 at 20 – 22 °C (Ha et al., 2010; Jin et al., 2016; Zhang et al., 2005). When newly transferred to a lawn of PA14, worms feed on the lawn. However, in the next few hours worms start to leave the lawn [Figure 2 and (Chang et al., 2011)]. The virulence of the bacteria, as well as several bacteria-derived chemicals act together to repel the worms from the lawn. The mechanisms underlying the changes in behavior and physiology of the worm over this process are separately reviewed (Hoffman and Aballay, 2019; Kim and Ewbank, 2018; Meisel and Kim, 2014) and (Flavell and Kim, this issue)].

Do worms learn to associate the aversiveness of PA14 with sensory cues produced by the pathogenic bacteria to generate retrievable memory of the bacterium? This question can be

addressed by testing the response to PA14-derived sensory cues in the naive, *i.e.* *E. coli*-raised, worms and the trained, *i.e.* PA14-fed, worms [(Ha et al., 2010; Jin et al., 2016; Liu et al., 2018; Zhang et al., 2005) and Figure 2A – 2F]. Previously, by probing the worms with an assay that resembles the standard chemotaxis assay on olfactory responses or with an assay that uses airstreams saturated with the odorants of tested bacteria, it is shown that after feeding on PA14 for 4 – 6 hours, the adult worms learn to reduce their preference for the odorants of the bacterium (Figure 2A) (Ha et al., 2010; Jin et al., 2016; Liu et al., 2018; Zhang et al., 2005). This type of learning in the adult *C. elegans* is contingent on the pathogenicity of the training bacteria and a serotonin signal. The training-dependent change in the olfactory response is specific for the odorants of the training bacteria (Figure 2A – 2C) (Choi et al., 2020; Ha et al., 2010; Jin et al., 2016; Liu et al., 2018; Zhang et al., 2005). Together, these results indicate a learned association between pathogenicity and the odorants of the training bacterium. Since pathogenic bacteria represent a critical constraint to the survival of *C. elegans*, it is conceivable that *C. elegans* has evolved the ability to associate the olfactory cues of some pathogenic bacteria with the virulence, which regulates subsequent interactions with the pathogen. The learned behavioral response is reversible, which suggests a temporary modulation of the nervous system (Ha et al., 2010; Jin et al., 2016; Liu et al., 2018; Zhang et al., 2005).

Several different methods have been used to analyze the changes in behavioral responses to pathogenic bacterium PA14 after feeding on PA14 [Figure 2 and (Ha et al., 2010; Hao et al., 2018; Horspool and Chang, 2017; Jin et al., 2016; Lee and Mylonakis, 2017; Liu et al., 2018; Ma et al., 2017; Meisel et al., 2014; Miller et al., 2015; Moore et al., 2019; Ooi and Prahlad, 2017; Singh and Aballay, 2019; Wolfe et al., 2019; Zhang et al., 2005)]. Some of these methods differ in the types of the sensory cues that they examine, the spatial and temporal patterns of the cues, and the behavioral strategies that they measure (Figure 2). For example, the behavioral strategies used to distinguish between two simultaneously present bacterial odorants (Figure 2C – 2F) are likely different from those used to respond to two alternating bacterial odorants (Figure 2B). The odorant gradient established by a point source is not linear (Tanimoto et al., 2017). Therefore, the size of the testing plate is important for the intensity and the spatial pattern of the bacterial odorants sensed by the worms (Figure 2C and 2D). The duration for which the testing bacteria were placed or grown on the testing plate or the temperature used to cultivate the testing bacteria generates different mixtures of the sensory cues (Figure 2E and 2F). In addition, separating the training process from the testing assay makes it possible to examine whether a retrievable memory is formed. Furthermore, the assay that measures the occupancy of a bacterial lawn grown on an assay plate likely measures sensory responses elicited by direct contacts of the worms with the stimuli, including metabolites that are not volatile and mechanical cues, in addition to olfactory responses (Figure 2). While worms feeding on a lawn of pathogenic bacterium PA14 gradually leave the lawn over time (Figure 2G and 2H), it usually takes longer for the worms to significantly leave the lawn than to learn to reduce the preference for the odorants of PA14. Because different molecular and neuronal apparatus are employed to detect, process and generate behavioral responses to these various types of sensory information, studies using these different assay conditions enriched our understanding of different pathways through which the worm interacts with pathogenic bacteria.

## Imprinting

Interestingly, training the worms by feeding on pathogenic bacteria during the first larval stage (L1) for 12 hours forms the aversive memory of the odorants of the pathogens that can be retrieved during the adult stage [Figure 1 and (Jin et al., 2016)]. This form of memory is comparable to the imprinted memory characterized in various vertebrate animals (Lorenz, 1935; Nevitt et al., 1994; Wilson and Sullivan, 1994). The worms imprint not only the odorants associated with the aversive experience, but also the odorants associated with food sources to form a long-lasting appetitive memory [Figure 1 and (Remy and Hobert, 2005)]. Mapping the neural circuits for the imprinting of pathogen odorants and the retrieval of the aversive memory, which take place two days apart, show that different circuits subserve learning and retrieval (Jin et al., 2016). In addition to the odorants that often represent food, pheromones also signal significant environmental conditions, such as the density of the conspecifics, to the worm (Butcher et al., 2007; Jeong et al., 2005; Macosko et al., 2009; Srinivasan et al., 2008; Srinivasan et al., 2012; White et al., 2007). Exposing the worms during the L1 stage to a repulsive pheromone enhances the avoidance of the pheromone during the adult stage by strengthening the synaptic connectivity between a pheromone-sensing neuron and its downstream motor neurons [Figure 1 and (Hong et al., 2017)]. Starvation during the L1 stage also profoundly alters the wiring of the nervous system by regulating neurotransmitters that respond to food availability (Bayer and Hobert, 2018). It is conceivable that during the early larval development when the nervous system is being formed, strong neuronal activities in response to environmental conditions reprogram the developmental process to generate persistent changes. A couple of studies show that harsh conditions during development systematically regulate gene expression and modulate the anatomy and activity of the nervous system, which produce behavioral changes that last into the adult stage. For example, when the worm density is high and food is relatively sparse, the larval worms can enter a diapause state, dauer, that halts the development for days until the conditions improve (Golden and Riddle, 1984). Adult animals that have experienced the dauer stage exhibit distinct behavioral traits, including those important for food seeking. The molecules that regulate chromatin structures and endogenous RNAi pathways mediate dauer formation, which potentially modulate the expression of genes underlying dauer-inducing changes in anatomy and behavior (Bharadwaj and Hall, 2017; Hall et al., 2010; Ow et al., 2018; Pradhan et al., 2019).

## Intergenerational effects

Because pathogenic bacteria serve as food sources and critical survival constraints to the worm, it is plausible that exposure to the pathogens modulates the nervous system and the behavior of the progenies. A recent study shows that training adult hermaphrodites by feeding on PA14 for 4 hours, which is known to produce a robust aversive memory that associates PA14 odorants with virulence in the adult mothers, increases the progeny's preference for the PA14 odorants (Pereira et al., 2020). Many animals prefer the food that they are exposed to *in utero* (Liu and Urban, 2017; Nehring et al., 2015; Todrank et al., 2011). These results suggest that with 4-hour exposure the food response elicited by PA14 is significant in the hermaphrodite mothers and that the resulting signals modulate the progeny developing in the uterus. Increasing the duration of PA14 exposure to 8 hours enhances the infection to the mothers (Troemel et al., 2006) and reduces the preference for PA14 in the



progeny (Pereira et al., 2020). These findings suggest that longer exposure to PA14 induces a stronger response to the pathogenicity of PA14, which changes the response of the progeny to PA14 from attraction to avoidance. While 4 to 8-hour parental training with PA14 produces robust aversive memory in the hermaphrodite mothers, their modulatory effects on the progeny are limited to the first generation of the offspring (Pereira et al., 2020).

Further increasing the duration of PA14 exposure to 24 hours that starts at the L4 larval stage not only generates avoidance of PA14 in the exposed mothers, but also produces PA14 avoidance in the progeny for 4 generations (Moore et al., 2019). Similar to the effect of 4-hour training on the progeny, the multi-generational effect requires the endo-RNAi pathway and the piRNA pathway (Moore et al., 2019; Pereira et al., 2020). Small RNA pathways also mediate olfactory adaptation by regulating the expression of genes critical for odorant sensation (Juang et al., 2013). However, these behavioral changes differ in their durations and modulatory effects, which suggest distinct mechanisms through which the underlying small RNA pathways alter the nervous system and behavior.

In addition to food-seeking related behavior, exposing the worms to pathogenic bacteria generates intergenerational effects on other physiological traits critical for survival. For example, exposure to *Pseudomonas vranovensis*, another bacterium pathogenic to *C. elegans*, generates multigenerational effects and enhances immune resistance to the pathogen in the offspring (Burton et al., 2019). Meanwhile, exposure to certain pathogenic bacteria for two consecutive generations induces formation of dauers, a dormant development stage that is highly resistant to environmental stresses (Palominos et al., 2017). These studies reveal multiple ways that the worm has evolved to adapt its development and function to the bacteria in its environment. Interestingly, pathogenic bacteria are not the only environmental conditions that impact the worm for multiple generations. It has been shown that parental experiences, including dietary restriction, osmotic stress, temperature changes, olfactory imprinting, and prolonged starvation, can regulate the physiology of the offspring, some of which last for several generations and are mediated by small RNA pathways (Burton et al., 2017; Burton et al., 2019; Das et al., 2020; Demoinet et al., 2017; Greer et al., 2011; Hibshman et al., 2016; Jobson et al., 2015; Klosin et al., 2017; Ni et al., 2016; Palominos et al., 2017; Posner et al., 2019; Rechavi et al., 2014; Remy, 2010; Schott et al., 2014; ). These studies together show that *C. elegans* has evolved diverse adaptive strategies to generate long-term plasticity that lasts for multiple generations. The short lifespan of *C. elegans* and the ease to conduct genetic experiments on it makes this line of research productive. It will be informative to compare the mechanistic insights identified in studies on intergenerational effects in different animals to understand the difference and similarity in the logic of these regulations.

## Outlook

*C. elegans* lives in a bacteria-rich environment that represents a vast amount of opportunities and constraints to its survival, reproduction and evolution. We are at the beginning stage to understand its impressive adaptive responses encoded in a compact genome. In addition to its responses to pathogenic bacteria, several recent studies also reveal interesting interactions between *C. elegans* and its commensal bacteria species. These studies show that *C. elegans*

utilizes neurotransmitters or vitamins produced by the environmental bacteria (O'Donnell et al., 2020; Urrutia et al., 2020; Wei and Ruvkun, 2020) to maintain or modulate various physiological events and neural functions. These findings together with those investigating the interactions of *C. elegans* and pathogenic bacteria have established *C. elegans* as a promising system to probe mechanisms underlying gut-brain interactions. Together, these studies allow us to leverage the experimental powers provided by a model organism to investigate the function of the nervous system in an ethological and evolutionary context.

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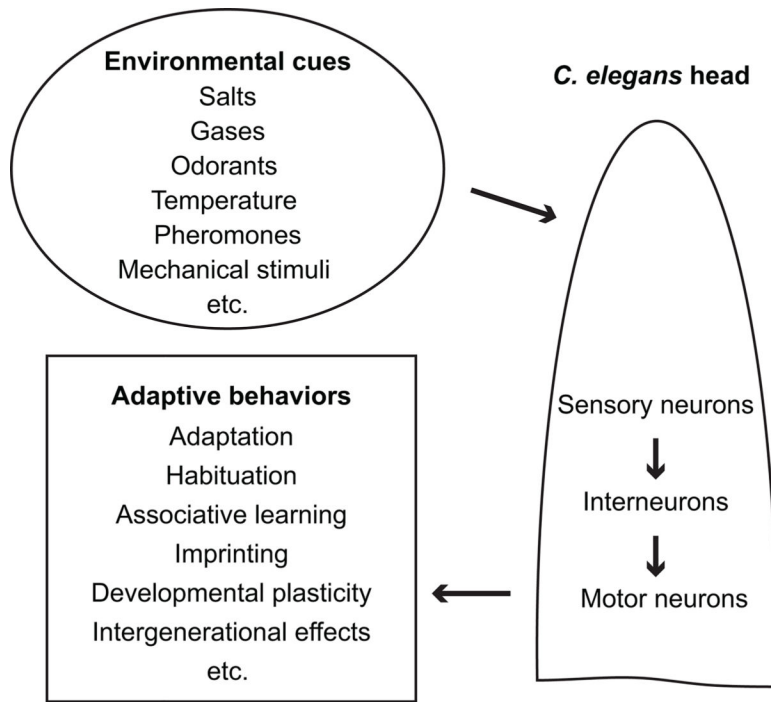
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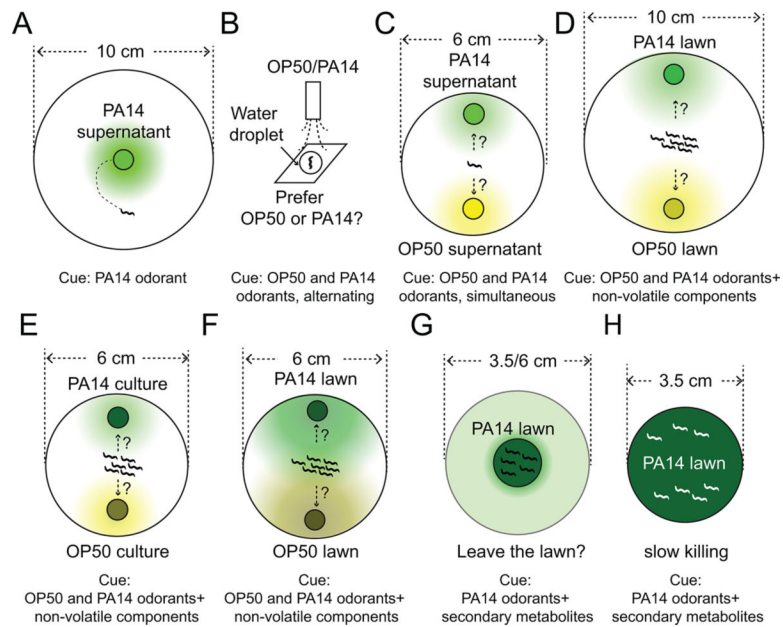
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**Figure 1.** Diverse adaptive behaviors in response to environmental cues in *C. elegans*.



**Figure 2. Schematic diagrams showing assays for behavioral responses to PA14.**

(A) A small drop of supernatant of PA14 culture is used as the source of odorants to examine attractive steering movements, when a worm starts from a position relatively close to the odorant source.

(B) To test the relative preference between the odorants of PA14 and the odorants of *E. coli* OP50, two airstreams saturated with the odorants of OP50 or the odorants of PA14 are used to deliver alternating stimuli to individual worms swimming in a small drop of buffer in an airtight chamber.

(C, D) Two small drops of supernatant of bacterial culture are put on a plate immediately before the assay (C) or two small drops of bacterial culture are quickly air-dried before the assay (D) to measure the preference between the two odorant mixtures in a single worm (C) or a population of worms (D). In D, the plate does not contain peptone and therefore does not support growth of the bacteria.

(E, F) A small plate with two bacteria lawns grown on the plate for a few hours (E) or for 24 hours (F) to be used as odorant sources. During cultivation, the bacterial lawns may produce cues diffused into the medium, produce or alter the concentration of gases in the lawn areas.

(G, H) A bacterial lawn centered on a small plate (G) or completely covering a small plate (H) prepared by fully growing first at 37°C and then at 25°C to examine the lawn avoidance/occupancy or survival of the worms over time.