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The Role of Nutrition in Regulating Aversive Behavior

A Major Qualifying Project Report

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Abstract:

All organisms are constantly interacting with multiple stimuli; sensing and processing them, which ultimately results in behavior. The internal processes which govern these behaviors are complex and remain largely unclear. The nematode *Caenorhabditis elegans* detects hundreds of small molecules to aid in navigating and surviving within its habitat, rendering it a strong model system for elucidating these mechanisms. These molecules may be generated by other species or by conspecifics. One class of molecule released by *C. elegans* are the ascarosides, which function as intraspecies pheromones. Octopamine Succinyl Ascaroside #9 (osas#9) is one such ascaroside which is released by starved young larvae, likely communicating/indicating that an area lacks food. When individuals sense this molecule, they avoid the area by undergoing an innate avoidance response, characterized by backing out and initiating a reversal motor program. However, in the presence of osas#9 and the microbial food source, *Escherichia coli*, *C. elegans* does not perform an avoidance response, suggesting that food negates the osas#9 aversive cue. This information served as a basis for testing which, if any, molecular metabolites released by *E. coli* attract *C. elegans* and negate the osas#9 avoidance response. A dose response curve was generated for the bacterial metabolite(s) with respect to osas#9, and the neuron responsible for detecting the metabolite(s) was identified. These results were used to analyze the neural basis of an innate choice-driven avoidance response.

Background:

The model organism *Caenorhabditis elegans* is frequently studied, however little is known about the mechanisms underlying how these animals navigate through their environment. It is known that some substances such as glycerol will elicit avoidance while others elicit attraction. However, the specific nature and mechanisms of these behaviors is not well characterized. In 1973, Ward hypothesized that cAMP and other various ions could play a role in the attraction to bacteria, but found that they did not under laboratory conditions[16]. Data from Sawin et al in 2000 suggested that bacterial lawn viscosity plays a role in food detection[11]. In 2004, Grey et al. looked at O_2 sensation and claimed it to be a factor in food finding behavior[5]. In 2014, Bassler et al. found that a 4-ketone and long carbon tail was detected by the *C. elegans* AWC neuron in order to interact with *v. cholerae*[17].

C. elegans use their amphid sensory neurons to detect water soluble and volatile chemical changes in their environment. The 12 sets of amphid sensory neurons and their

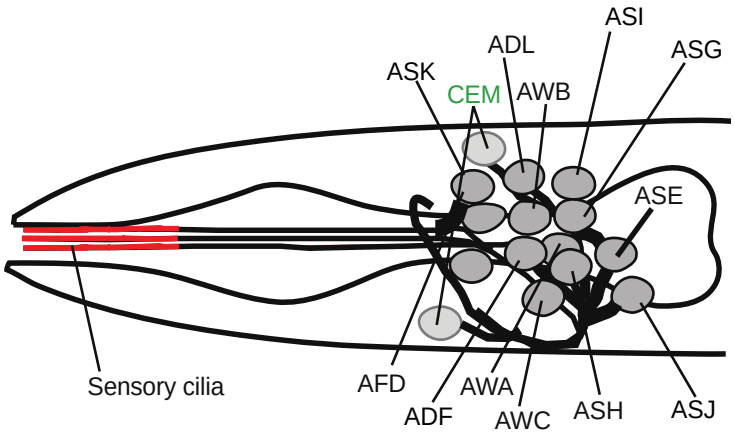

Figure 1: ***C. elegans* Sensory Neurons** (Adapted from Chute and Srinivasan, 2014)[4]

corresponding cilia are located in the head of the animal (Fig. 1). Of these 12 sensory neurons, ASE, ASG, ASI, ASK, AWA, and AWC are of particular interest as they are associated with chemoattraction[12]. A limited list of chemical attractants is known (Table 1), but the full list of detectable molecules is still far from complete.

Table 1: **Known Attractant Molecules for *C. elegans*** (Adapted from *C, elegans* II 2nd Edition)[10]

Attractants	
Water-soluble	Volatile
Na^+, K^+, Li^+ Ca^{++}, Mg^{++}	alcohols
Cl^-, SO_4^-, NO_3^-, Br^-, I^-	ketones
cAMP, cGMP	diketones
Biotin	esters
Lysine, histidine, cysteine, serotonin	pyrazines
Basic pH	thiazoles aldehydes aromatics ethers

In addition to substances that are naturally in the environment driving behaviors, *C. elegans* use small molecules known as ascarosides to communicate with each other. Ascarosides are

composed of an ascarylose sugar base with a wide range of constitutive moieties ranging from lipid side chains to bioamine derivatives (Fig. 3). The myriad of combinations and alterations appears to be like an alphabet, communicating specific behavioral and social signals. This modularity allows for a high flexibility of possible molecules while limiting the number of biochemical pathways that are needed for synthesis[8]. For example, Ascaroside #3 (ascr#3) is a hermaphrodite repellant ascaroside that contains a base ascarylose attached to an α,β-unsaturated fatty acid. If the fatty acid is saturated, then it is instead known as ascaroside #10 which attracts hermaphrodites[7]. Likewise, an addition of a catecholamine communicates a completely different signal. Important to this study is the aversive response elicited by the bioamine derived ascaroside **O**ctopamine **S**uccinyl **AS**caroside **#9** (osas#9). It is released by L1 larvae that are starving, likely as a byproduct of the metabolic and physiological changes resulting from starvation[1]. When individuals encounter osas#9, they back away from the area, displaying a characteristic reversal avoidance response. This suggests the molecule is a

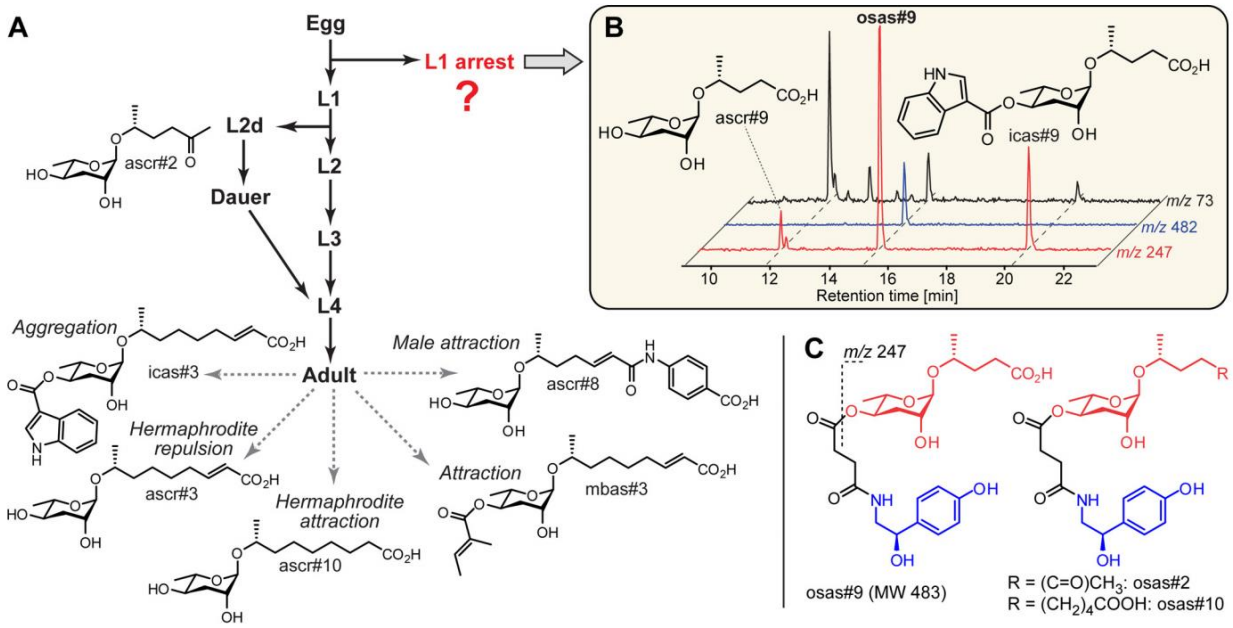

Figure 3: **Ascarosides in *C. elegans* and detection of ascarosides specific to starved L1 larvae** Examples of different ascarosides including osas#9 (Adapted from Artyukhin et al. 2014)[1]

possible ecological cue to conspecifics, communicating the condition of the habitat.

Further supporting osas#9's role as a food availability cue, is the fact that it can be attenuated by the presence of food. Artyukhin et al. found that avoidance elicited by osas#9 was cancelled out by the presence of *E. coli*. In fact, baseline avoidance to osmolarity differences was also cancelled out. This led to the conclusion that responses to these ascarosides can be flexible and that there is at least one small molecule metabolite produced by *E. coli* that attracts *C. elegans*[1]. Attenuation of the avoidance of osas#9 could then be used as a tool to determine what metabolite is responsible, what neuron is sensing it, and ultimately how relevant information is being integrated. This could then ultimately lead to the understanding of how the worm considers multiple inputs when making decisions.

Methods:

Strains:

Wild-type animals were *C. elegans* var. Bristol, strain N2 obtained from the *Caenorhabditis* Genetics Center. Genetically ablated strains were obtained from the Wakabayashi lab (ASK strain PS6022) and the Department of Biology and National Center for Behavioral Genomics, Brandeis University (AWC strain PY7502 and ASI strain PY7505). Genetic ablations were achieved though neuron-specific recCaspase expression[2]. Worms were grown under standard conditions at 20 °C on a lawn of OP50 bacteria[14].

Drop Assay:

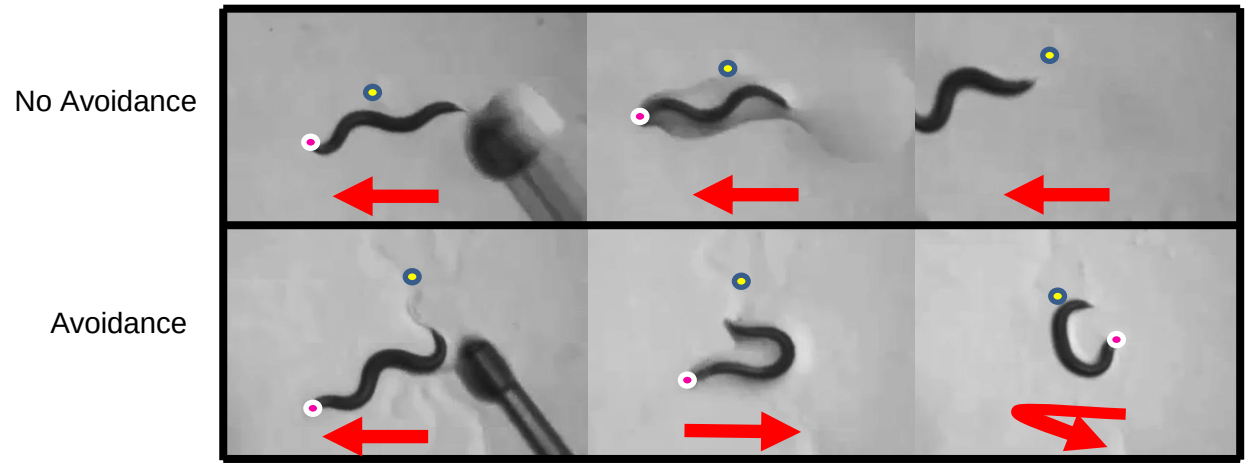

Figure 4: **Possible *C. elegans* Drop Assay Outcomes.** Red arrow indicates direction of movement. White/red dot indicates amphid sensilla. Blue/yellow dot represents an unmoving point of reference. A "No Avoidance" response is characterized by the treatment liquid surrounding the worm but eliciting no response. An "Avoidance" response is characterized by backing out and a subsequent reversal motor program (Adapted from Chute)

A drop assay was used to assess the behavioral response of the worms to various treatments (Fig. 4). Worms were washed off of a 5cm plate in M9 solution and re-plated onto an unseeded 5cm plate. After 4 hours, individual worms had a drop of solvent control placed behind them in such a way that it surrounded their body and reached their amphid cilia without triggering a touch avoidance response. An avoidance response was defined as a reversal, omega turn, or >90°turn. 15 or 20 worms were assayed per plate, and a total **A**voidance **I**ndex (AI) was calculated by dividing the number of avoidance responses by the total number of drops[16].

$$(\text{AI} = \frac{drops\ avoided}{total\ drops}).$$

A solvent control is tested prior to testing the treatment in order to account for any baseline avoidance that may occur due to osmolarity, temperature, or solvent composition. This baseline avoidance is subtracted from the treatment Avoidance Index to obtain the **N**ormalized **A**voidance **I**ndex (NAI)

Bacterial Extract:

The bacterial extract was obtained from the Schroeder lab (Cornell) and was dissolved in 100% EtOH. Ten-fold serial dilutions in 100% EtOH were then used to achieve varying extract concentrations. In order to create the solution for use in the drop assay, 11 µL of 10 µM osas#9 dissolved in 100% EtOH (to achieve a physiologically relevant concentration) and 1 µL of corresponding extract were added to 98 µL of DI water and given time to equilibrate to room temperature. For example, to create a solution for testing a concentration of 1/1000 extract, the following solutions were combined: 11 µL of 10 µM osas#9 dissolved in 100% EtOH, 1 µL of 10% extract, and 98 µL of DI water. At least three days of data were used for each extract concentration.

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Laser Ablations:

Agar pads were made by placing 200µl of 4% noble agar in M9 salt buffer with 20 mM Sodium Azide (NaN_3) on a microscope slide. Additionally, 0.6 µl of 25 mM NaN_3 was placed on the agar pad and 1-3 L1 N2s were placed in the NaN_3 drop. A slide cover was placed on the pad, and the slide was observed under a 100x oil immersion on a compound scope with a Micro Point laser attachment. Target cells were identified using a map of neurons and killed using a medium intensity setting on the laser. Successfully ablated worms were transferred to seeded plates. Mock ablations were performed under the same conditions but without firing the laser.

Statistics:

Prior to performing statistical comparisons for any data set, normality was assessed using a D'Agostino & Pearson omnibus normality test. Fig. 5A did not pass so a nonparametric Mann-Whitney test was performed resulting in a P value of 0.0006 (N = 7). All subsequent data set passed a D'Agostino & Pearson omnibus normality test, so parametric one-way ANOVAs were used. In order to obtain p values when comparing multiple sets, Tukey's Multiple Comparisons test was used.

Results:

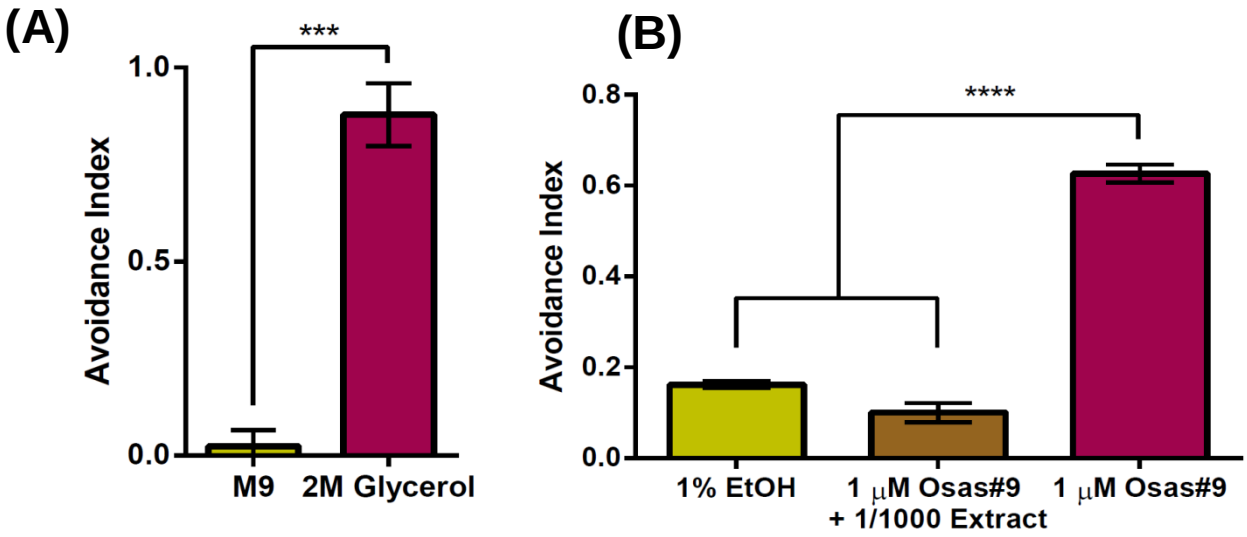

Fig 5. (A) Avoidance to M9 solvent and 2M Glycerol. (P=0.0006, N=7) P-value obtained from Mann-Whitney test. Error bars represent SEM. **(B)** Avoidance to 1 µM osas#9, 1% EtOH solvent, and 1 µM osas#9 + 1/1000 bacterial extract (P<0.0001). P-values obtained through one-way ANOVAs using Tukey's Multiple Comparisons test. Error bars represent SEM.

Initial avoidance assays were performed using 2M Glycerol (Fig. 5A) which is known to cause nearly 100% avoidance[6]. Once the drop amount for the assay was "calibrated", a baseline for 1 µM osas#9, 1% EtOH solvent, and 1 µM osas#9 + 1/1000 bacterial extract were generated (Fig. 5B). A Normalized Avoidance Index may be obtained by subtracting the 1% EtOH solvent control from the corresponding treatment Avoidance Index on a plate by plate basis in order to account for interplate variation. All subsequent figures use Normalized Avoidance Indices.

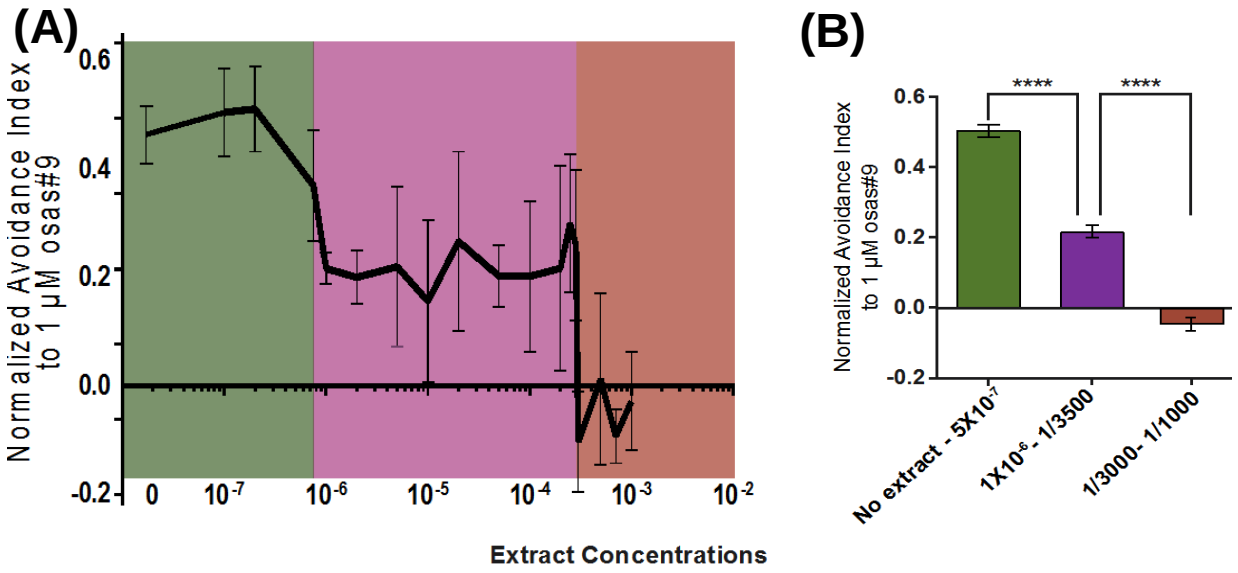

Fig 6. (A) Extract concentration on a continuous logarithmic scale vs. normalized avoidance to 1µM osas#9 **(B)** Extract concentration divided into three ranges vs. normalized avoidance to 1µM osas#9 ($P<0.0001$). All P-values obtained through one-way ANOVAs using Tukey's Multiple Comparisons test. Error bars represent SEM. Negative NAI is the result of higher avoidance in solvent control than treatment. Colors correspond between figures.

The extract response curve (Fig. 6A) is made up of three distinct behavioral responses. There is no clear pattern of increase or decrease within each concentration range, the results show two thresholds that each demarcated three independent response types. The three concentration ranges were divided into bins (Fig. 6B) and were determined to be statistically significant ($P<0.0001$) from each other.

The responses to solutions containing extract concentrations that were lower than the 5×10^{-7} dilution (**Green Region**) were statistically insignificant ($P>0.05$) from responses to solution with no extract. The responses to solutions containing extract concentrations in the range from 10^{-6} to 3×10^{-4} (**Pink/Purple Region**) were characterized by high variation; with some plates being similar to no extract while other plates being similar to undiluted extract. The responses were not bimodal however, as there were also plates that were halfway between the two. Extract concentrations higher than 3×10^{-4} (**Brown Region**) resulted in a complete attenuation of the avoidance response, with most NAI values being 0 or less.

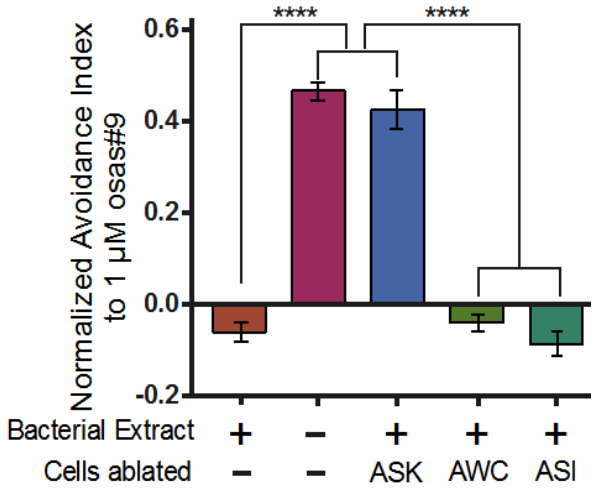

Figure 7: **Response to osas#9 by genetically ablated animals** (P < 0.0001). P-values obtained through one-way ANOVAs using Tukey's Multiple Comparisons test. Error bars represent SEM. Negative NAI is the result of higher avoidance in solvent control than treatment.

The data indicates that ASK genetically ablated worms responded to osas#9 with an avoidance response, even in the presence of food (bacterial extract) (Fig. 7). Contrarily, two other suspect neurons, AWC and ASI, showed attenuated responses to osas#9 in the presence of food which resembles the phenotype of wild type worms. Therefore, these sensory neurons are not required for the food sensing response.

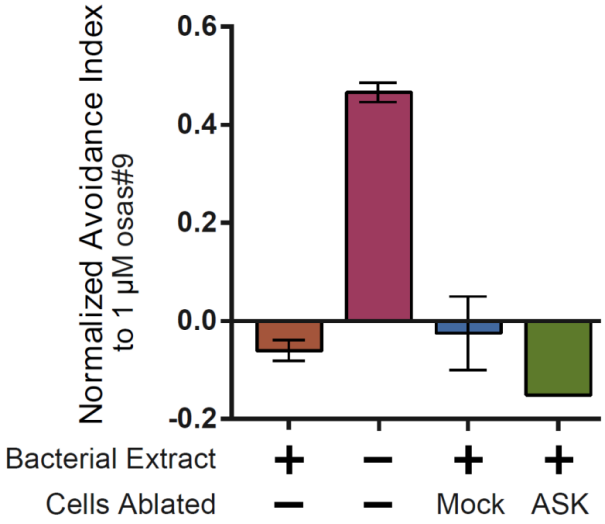

Figure 8: **Response to osas#9 by laser ablated animals.** N was too low to perform statistical analyses.

To confirm ASK's role in sensing the metabolite necessary for attenuation, laser ablations were performed (Fig. 8). The data obtained from these laser ablation experiments were not replicated (n=1) and contradict the data from the genetic ablation experiments.

Subsequently, it was of interest to determine the metabolite in the extract responsible for this attenuation. Niacin and Nicotinamide together constitute up to 10% of the bacterial extract. Therefore, they were the primary candidates tested.

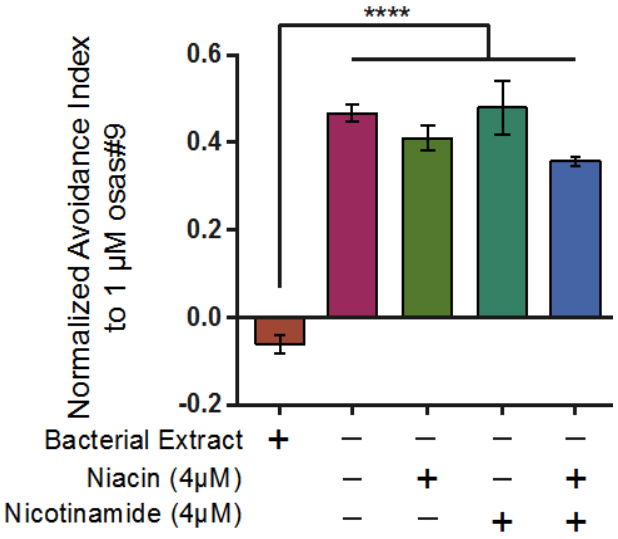

Figure 9: **Attenuation of the avoidance response by bacterial extract components** ($P<0.0001$). P-values obtained through one-way ANOVAs using Tukey's Multiple Comparisons test. Error bars represent SEM. Negative NAI is the result of higher avoidance in solvent control than treatment.

It was found that neither Niacin nor Nicotinamide were responsible for attenuation (Fig. 9). Furthermore, the possibility of synergy was explored, however, the combination of the two compounds did not attenuate the response to the extent that full extract did. The difference between the no extract group and the Niacin + Nicotinamide group was statistically significant, but only slightly ($p = 0.0481$)

Discussion:

While previous data[1] showed that whole *E. coli* attenuated the avoidance response, these results confirm that only the extract of the bacteria is needed. This means that *C. elegans* do not sense *E. coli* by detecting a physical property (such as a surface protein) and instead use a molecular, chemosensation, detection method. The results show that *Escherichia coli* metabolites attenuate the avoidance response to osas#9. This is further evidence that osas#9 is a molecular cue of unfavorable habitats in relation to food availability. While *E. coli* is not a common food for *C. elegans* in the wild, it is likely that the metabolite is from a pathway that is common to soil-dwelling bacterial. Regardless of whether it would encounter the metabolite regularly, its attenuation of the osas#9 avoidance response allows the effects of varying metabolite concentration to be studied.

Attenuation is dependent on concentration. There are three different ranges of concentration that result in separate behavioral outputs. The first occurs when there is a high amount of food, up to a 1/3250 dilution (**Brown Region**). Normalized avoidance is at or less than 0 and the worms often prefer the extract much more than the solvent control, even in the presence of osas#9. This is in contrast to when no or little food is present (down to a one-billionth dilution) (**Green Region**) and avoidance is approximately 0.5. Between the two is a mid-response range that is approximately half of the avoidance of no food (**Pink/Purple Region**). It consists of high variation due possibly to the state-dependence of the worm when being assayed. Relative age (hours difference) and physiological state likely plays a role in the variance seen in the intermediate range. These concentrations are not weak or strong enough for a robust response, and it is likely that the response is more sensitive to the exact state of the worms tested. The underlying mechanisms driving this “thought process” are still unknown. For instance, the dual threshold phenomenon seen in the extract response was not expected. Typically, a ligand-receptor binding relationship exists as a single logarithmic curve. It is possible that the middle response occurs when a small number of molecules are binding and the full response only occurs once the receptors are saturated. Another option is that there is one metabolite that is easily detectable but does not attenuate avoidance as much as another less easily detectable metabolite. Both situations are just as plausible and require further research. Despite this, the sensory neuron that initially detects the metabolite was identified, providing the necessary basis to unravel the specific mechanisms.

The ASK sensory neuron is required for sensing food in *C. elegans*. Laser ablation experiments were performed (Fig. 8) but resulted in poor results due to novice ablation skill and a low n. ASK was identified as the food sensing neuron primarily through the genetic ablation experiments (Fig 7.) as well as a paper that was published concurrently with this project[3]. ASI and AWC are not required for food sensing. This was surprising, as AWC is a neuron known to play a role in enacting many chemoattraction behaviors, including a possible food finding response[17]. However, ASK fits the model better due to a gap junction with ASH, the sensory neuron required for detecting osas#9 (Srinivasan Lab, unpublished data). It is possible that there is direct inhibition of ASH or further downstream inhibition (Fig 10). Calcium imaging techniques (G-CaMP) can be employed to fully characterize the exact interactions between the

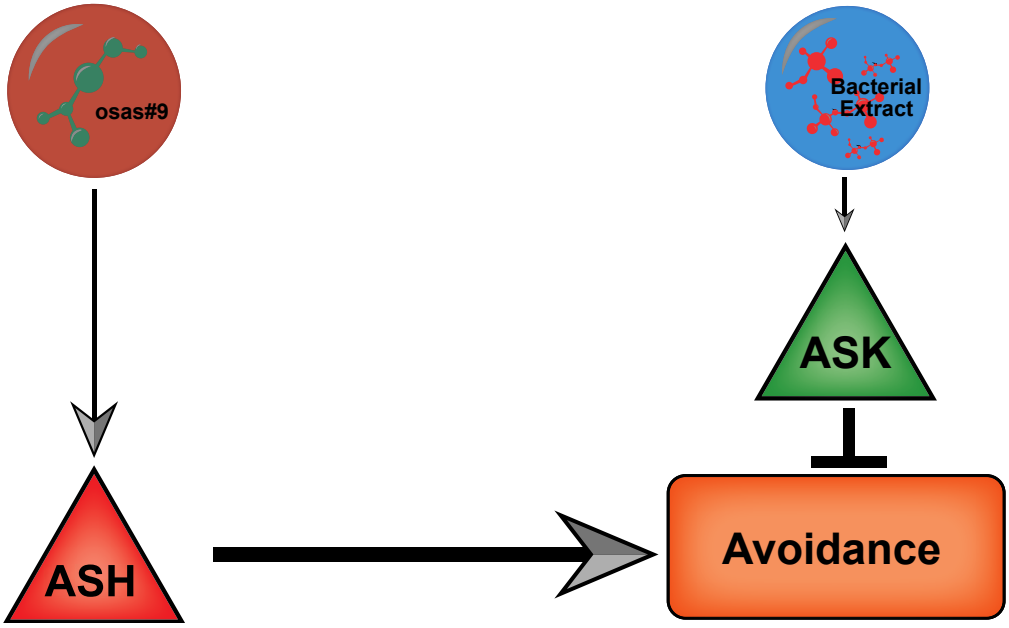

Figure 10: **Working Model of Interaction between ASK and ASH sensory neurons**

pathways. In order to better characterize the pathway, it would be necessary to identify the receptor responsible for binding the metabolite. The metabolite must first be identified in order to accomplish this.

Niacin and Nicotinamide are the most prevalent constituents of the bacterial extract, together making up roughly 10% of it. This made them the first candidates for testing. Individually they show absolutely no attenuation at physiologically relevant concentrations. However, the combination of the two showed a slight synergistic affect, partially dampening the response. This affect was minimal and only marginally statistically significant (p=.0481). They do not attenuate the avoidance response as nearly as much as the extract does. This demonstrates that they are not solely the molecule that *C. elegans* detect in the extract and it is most likely a synergistic affect. It is possible they could work in conjunction with another metabolite, and this requires further study and characterization. This is not uncommon in the worm, in fact mate attraction cues are known to be synergistic, requiring several ascarosides to reconstitute a wild type phenotype[13].

The attractant could possibly be implied by looking at a list of molecules that ASK detects. One molecule which is indicative of a food-finding response that is detected by ASK is L-Lysine[15]. L-Lysine is an essential amino acid for the worm which further validates its candidacy[9]. If L-Lysine is not responsible for the attenuation, then further fractions of the extract would need to be tested.

Conclusions:

Every organism with a nervous system takes multiple stimuli into account when making decisions. Innate behavioral reactions to specific stimuli can increase the chances of survival in a hostile environment. The straightforward, deterministic nature of these innate behaviors makes them reproducible and as a result they are excellent tools for studying behavior as a whole. By studying a simplified nervous system with a low number of possible inputs, it is easier to understand the underlying mechanisms driving choice-based behaviors and will ultimately lead to understanding the complex machinations of the human mind.

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Strains:
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Wakabayashi Lab
Department of Biology and National Center for Behavioral Genomics, Brandeis University

Previous Work:

Work was performed during A and B terms in Prof. Mark Alkema's lab at the University of Massachusetts Medical School. The goal of the project was to use a hyperactive gain-of-function calcium channel to create *C. elegans* with a heightened sense of smell. This mutation is homologous to a mutation found in humans with chronic migraines, and understanding the way it affects specific cells could lead to a further understanding of how these migraines could be treated. Unfortunately, the project was discontinued before C term due to scheduling conflict.

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