Caenorhabditis elegans experiences leave behavioral modifications on great-grandchildren

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Abstract:

The study of epigenetics explores the idea of how one’s behavior is due to their DNA or the way they are raised. We developed a paradigm to test behavioral epigenetics in the microscopic nematode, *Caenorhabditis elegans*. *C. elegans* serve as a great genetic model organism because they have a short life cycle, roughly 1000 cells and a third of those being neurons. We hypothesized that pre-exposure to a biogenic aversive cue produced aversive stimuli, octopamine-succinylated ascaroside #9 (osas#9), would result in a decreased sensation to the chemical across multiple generations. Our results show that pre-exposure to osas#9 results in decreased aversive response that is observed across three filial generations. However, the procedure was repeated with a non-native aversive stimulus, glycerol, and showed that there was no memory in the parental or any of the three filial generations. Our data suggests that *C. elegans* have an epigenetic memory to biologically-produced stimuli, but not non-native aversive stimuli, across multiple generations and exemplifies how nurture can later affect nature.
Background:

Epigenetic memory

Have you ever wondered if the way you are raised will affect your behaviors growing up? Maybe you would behave differently if your parents spent less time with you and neglected you, if you grew up in a richer neighborhood, or if you grew up short on money and could not afford food. This is the idea of nature versus nurture; is it your genes or surroundings that influence your behavior (Mcleod, 2015)? Biologists have started researching this concept of nature versus nurture through the study of epigenetics, seeing if the way you are raised can cause DNA modifications and affects gene expression (Cowell, 2012). Currently, epigeneticists are looking at these gene modifications and seeing if the modifications could be due to the way one is raised, and if so, determining how the modifications affect the behavior of their children, or even their great-grandchildren (Hurley, 2013).

Epigenetic modifications can change gene expression, however they do so without affecting the DNA sequence. Instead, small chemical tags are added or taken away from the DNA, changing a gene's likelihood of being expressed. In humans, the two chemical tags that are most common for DNA modification are methyl and acetyl groups. These groups can be added to one of the four bases of DNA (adenine, guanine, cytosine, or thymine) and modify the bases without changing the DNA sequence, meaning all genes are still transcribed the same. These modifications can have several effects including loosening or tightening the DNA around histone proteins. The tighter the DNA is wrapped around histones, the less likely it will be transcribed and translated into proteins. Modifications can also turn genes on or off by the process of imprinting. A human receives twenty-three chromosomes from the mother and twenty-three chromosomes from the father; however, both parental copies are not expressed for every gene. Either the mother’s or father’s gene can be turned off by methyl modifications (Cowell, 2012).

After understanding the molecular biology of modifications and epigenetics, scientists have looked to see if the environment can induce epigenetic modifications and if these epigenetic modifications can be passed on from one generation to the next. One such study found that people who had undergone abuse as children had more methylation on their DNA than people that had not undergone any form of abuse (Hurley, 2013). This showed that the way one was raised caused modification to their genome. Additionally, they found that those with higher abuse and methylation also had higher suicide rates, showing that the DNA modifications due to the
environment in which they were raised had an effect on their behavior (Hurley, 2013). Another study looked at children that were conceived during the famine of the Second World War in Europe, versus those conceived after the famine. The results were shocking and showed that children in the womb during famine had lower methylation levels of a particular gene, IGF2, leading to a higher likelihood of lifelong health problems (Cowell, 2012). This study in particular identified a very brief timeframe in development where epigenetic modifications can occur. Together, these two studies helped strengthen the correlation of parents’ behavior having lasting effects on their offspring.

Human studies are hard to perform, due to ethical considerations as well as the long time frame needed to carry out a clinical study, so scientists have looked towards using model organisms. One of the first studies done which truly showed the environment one is raised in can cause epigenetic modifications and affect one’s behavior was done in rats. In the 1980s, Dr. Syz and Dr. Meaney wanted to see if good versus bad parenting skills can affect the offspring’s behavior. To test this, they set up three different experiments. The first was simple, consisting of having an attentive mom that licked her pups and an inattentive mom that did not give the pups the same level of attention. They then let the pups grow up and assayed their behavior, finding that pups from the inattentive mother were very skittish compared to pups from the attentive mother (Hurley, 2013). A new hypothesis was developed proposing that this behavior was due to a genetic difference between the rat mothers. For the second experiment they took pups from the inattentive mother and gave them to the attentive mother to raise, and vice versa for the attentive mother’s pups. The data was striking and showed that the pups raised by the inattentive mother were more skittish, even though they had the genetics from the attentive mother (Hurley, 2013). This result was not genetic, so what was the cause? They set up a third experiment very similar to the first where they had the inattentive mother raise her biological pups which were very skittish, but then injected the pups with a drug called Trichostatin A, which removes methyl groups. After this injection, the pups all appeared normal and calm compared to their previous skittish behavior (Hurley, 2013). This study showed that the way the rat pups were raised caused DNA modifications, or epigenetic changes, which affected the rat pups’ behavior.

Animal Behavior

The study of animal behavior seeks to answer questions pertaining to why animals behave in the ways they do. It integrates an animal’s anatomy and physiology and seeks to understand
how these are implicated in the resulting behavior. Animal behavior also explores how genetic and environmental factors, known as internal and external stimuli, prompt behavioral response (Hager, 2010). Scientists study animal behavior in context of ecology and evolution, with the goal of uncovering how animal’s interactions with their environment change over time (Hager, 2010). The study of non-human animal behavior can be useful in uncovering the underpinnings of human behavior and can aid in advances to improve human life (Hager, 2010).

Behavior is defined as action in response to stimuli. All behaviors serve a larger purpose, to help an animal avoid predators and unfavorable environments, to find food, and to find mates. Natural selection acts on behaviors as it does on physical traits, favoring animals that perform behaviors which make them more fit for survival. In order to understand behaviors, it is necessary to understand what stimuli cause the behavior, and what function the behavior serves (Ryan & Wilczynski, 2011). Ethologist Niko Tinbergen was interested in understanding how behaviors occur as well as why natural selection favors certain behaviors. He explored both proximate and ultimate causes of behavior, receiving the Nobel Prize in 1973 for his work in this area (The Nobel Foundation, 2014). Proximate causes of behavior encompass what stimuli cause the behavior to occur and what the animal’s body does in response to the stimuli (Mayr, 1988). Ultimate causes of behavior encompass what about the behavior helps the animal to survive, as well as the evolutionary history underpinning the continuation of this behavior (Mayr, 1988). Understanding animal behavior requires taking into account an integration of these proximate and ultimate causes, as well as considering molecular, hormonal, and neural control of the behavior (Ryan & Wilczynski, 2011).

Genes and Animal Behavior

An animal’s environment and genetic makeup can also shape behavior, influencing its development and evolution. Genes can influence the morphology and physiology of an animal in addition to creating the framework for cognition, learning, and memory. Instinctive behaviors are genetically determined, hard-wired, and cannot be modified by learning. An example of such instinctive behavior is a cockroach retreating to a dark corner when exposed to light; this action does not require learning or thought but is an innate response to specific stimuli within a specific context (Breed and Sanchez, 2010). Variation in genomes, gene variants, and gene expression can all contribute to difference in behavioral response. The presence of different genes within
individuals or species can lead to differences in behavior; this has been shown as a determining factor in behavioral taste preference for *Drosophila* species. Gene variants, or different alleles of shared genes, have been shown to impact behaviors such as courtship song frequency among many species of *Drosophila*. Variation of the expression of a set of genes can lead to differences in animal behavior within a given species or even within an individual over time. Up- or down-regulation of genes can lead to different phenotypes which can, in turn, inform particular behaviors (Ryan and Wilczynski, 2011). The environment can influence both morphological and physiological development, and can modulate animal behavior (Ryan and Wilczynski, 2011). A large component of animal behavior in the context of environment has to do with learning and responding to local conditions. For example, the spread of urbanization has induced a shift in foraging patterns for animals to maximize their odds of surviving and reproducing with an increase in human presence (Wong and Candolin, 2015). Changing landscapes and shifts in the availability of resources can lead to behavioral evolution.

**Animal Communication**

An important application of animal behavior is the ability to communicate effectively with other individuals, both within and outside of the same species. Communication between individuals can allow for successful location of food and favorable habitats, warning of nearby predators, attraction of mates, among other necessary behaviors (Gillam, 2011). This communication occurs through signal transduction and signal perception, with the signal either benefiting only the sender or the receiver, or benefiting both equally (Gillam, 2011). When communication between two individuals benefits both equally, this communication can be deemed altruistic. Altruism has been explained through the process of natural selection, as common interests drive the communication, and the result of signal reception allows for increased survival and reproductive success (Zahavi, 1977). One such example of altruistic communication occurs in the nematode, *Caenorhabditis elegans*, in response to lack of resources. Larval stage 1 (L1) *C. elegans* secrete a chemical signal when they are in an environment that lacks food and they become starved. The chemical signal, when sensed by other *C. elegans* nearby, causes dispersal of the worms and avoidance of the unfavorable environment (Artyukhin et al., 2013). Common interest for avoiding starvation and increasing chance of survival was a possible cause for this altruistic trait in *C. elegans* and could explain why this trait has evolved within the nematodes.
**C. elegans as a good model organism for Epigenetics**

Many animals can be used as a model organism including rats, mice, and pigs. However, our lab focuses on using *Caenorhabditis elegans* for behavioral studies. *C. elegans* are nonparasitic, microscopic nematodes that are naturally found in the soil. Figure 1 below provides an image of an adult *C. elegans*, which is about 1-1.2 mm in length. *C. elegans* have an extremely fast life cycle, consisting of four larval stages, a young adult, and an adult stage (WormAtlas.2008). The youngest larval stage is called L1 and goes all the way up to L4 before becoming a young adult (YA) and then an adult. Within three days, an egg is able to hatch and grow up to be a young adult. This fast life cycle allows for assays to be completed within a few days to a week, allowing for large-scale data collection in a short time-frame (WormAtlas, 2008).

![Image of C. elegans](image)

**Figure 1. Adult N2 C. elegans.** Obtained from The Chin-Sang Lab

In addition to the fast life cycle, the entire genome of *C. elegans* was mapped out in 1998, making it the first animal to have its genome fully sequenced (The C. elegans Sequencing Consortium, 1998). This sequencing provided key information that can be used to help aid in the understanding of gene function. *C. elegans* has about 19,000 genes which encode for 1000 cells. Of these 1000 cells, 302 are neurons and 56 are glial cells, which comprises a third of the animals cells (WormAtlas, 2008). The *C. elegans* nervous system is relatively simplistic, but still allows the animal to respond to stimuli and have complex behavioral outputs.

It is important to note that *C. elegans* can’t see or hear, they can only communicate through smell and taste, known as chemosensation (WormAtlas, 2008). Chemosensation refers specifically to the sensation of chemicals or odorants found naturally in their environment or produced by other *C. elegans* (Chute & Srinivasan, 2014). The chemicals or odorants produced by *C. elegans* are known as ascarosides. Ascarosides act as pheromones, with modular differences in these ascarosides acting as the animal’s own “alphabet”, or way to communicate different information with each other.
Recently, there have been multiple epigenetic studies where *C. elegans* have been employed as the model organism. A cross-study performed on humans and *C. elegans* successfully utilized conserved genes regulating sleep in *C. elegans* to show that sleep duration in humans may be due to differential DNA methylation (Huang et al., 2017). Thirty-four gene orthologs, genes present in different species which evolved from a common ancestor, controlling sleep were identified in the *C. elegans*. Decreasing the DNA methylation at a particular site in one of these orthologous genes correlated with shorter sleep duration in *C. elegans*, suggesting that differential DNA methylation in the human gene orthologs could also show this correlation (Huang et al., 2017). Utilizing *C. elegans* for the majority of the epigenome-wide methylation analysis was deemed more efficient and ethical due to the fast life cycle of the nematode and the easy accessibility for repeat experimentation. This study was unique in that it explored epigenetic modifications across species, allowing data from the study to be applied to both *C. elegans* and humans.

Another epigenetic study showed an environmental influence on gene expression in the progeny of exposed animals, with an inheritance pattern spanning multiple generations. A temperature-induced change in the expression of a *C. elegans* heterochromatic gene array was shown to endure for a minimum of 14 generations, indicating that *C. elegans* are a good intergenerational epigenetic model organism. *C. elegans* grown at 25°C were shown to have elevated expression of a promoter for heat shock protein constructs; this elevated expression was sustained in the progeny of these worms once they were transferred to grow at 20°C, but was not sustained for as many generations as expression from the integrated multicopy array. The authors of this study speculate that inheritance of changes in expression from repressed chromatin, induced by a shift in temperature, could indicate co-option to provide adaptive benefits to *C. elegans* (Klosin et al., 2017).

A third study utilizing *C. elegans* as the model organism sought to uncover how epigenetic memory is transgenerationally inherited. By inducing starvation-based L1 developmental arrest in *C. elegans*, generation of small RNAs was observed and inherited for at least three subsequent and consecutive generations. These small, transgenerationally inherited RNAs were shown to target genes involved in nutrition pathways, allowing for discovery of the specific genes necessary for the observed multigenerational effect. Additionally, three generations of progeny of starved parents were shown to live longer, indicating future studies will need to be performed to understand
the connection between small inherited RNAs and heritable increase in longevity of *C. elegans* (Rechavi et al., 2014). The increase in longevity could prepare the progeny to face similar starvation or provide some other adaptive benefit, again indicating the benefits of epigenetic memory to increased survival of a species.

**Chemical/behavior assay**

As previously mentioned, *C. elegans* communicate through the use of chemosensation, smelling and tasting odorants known as pheromones. Chemosensation can be used to help with development or to help other animals find food. The sensation of these pheromones is completed by the use of sensory cilia in *C. elegans*. Sensory cilia are partially exposed to the environment, allowing them to detect chemicals from the environment and send this information to the correct neuron (Bargmann, 2006). One type of specific chemicals detected by sensory cilia is called an ascaroside.

An ascaroside is a glycolipid naturally produced by *C. elegans* and released into the environment (Ludewig & Schroeder, 2013). The main component of an ascaroside is the ascarylose sugar that serves as the base of the molecule. From there, different fatty acid side chains can be added to modify and change the function of the ascaroside (Ludewig & Schroeder, 2013). There are many different varieties and purposes for ascarosides, but the one our project is focused on is octopamine-succinylated ascaroside #9, or osas#9, which is pictured in Figure 2 below. Osas#9 is produced by L1 *C. elegans*, the youngest life stage, when they are starved and signals to other animals that no food is present (Artyukhin et al., 2013). Animals sensing this chemical will reverse their body and avoid the area, an altruistic and evolutionarily beneficial behavior.

![Figure 2](image-url)

**Figure 2. Octopamine-succinylated ascaroside #9 (osas#9) structure.** Ascaralose sugar strucutre with a lipid side chain and octopamine moeity. Adapted from Christopher Chute.
For our project, we want to see what happens with repeated exposure to osas#9. It is hypothesized that repeated exposure to osas#9 will cause a change in *C. elegans* avoidance behavior, with the animals either avoiding more or less. It is also hypothesized that this change in behavior will be an epigenetic modification that can be passed on from one generation to the next. To test this, wild type N2 *C. elegans* were either pre-exposed to osas#9 or left unexposed and naïve. Their avoidance was tested after exposure and compared to see if any differences were noted. The animals were then carried on to the third filial (F₃) generation and avoidances across all generations were compared.
Methodology:

The goal of this project was to develop a paradigm for assessing an epigenetic memory to aversive stimuli in *C. elegans*. Several paradigms were designed, tested, and are described in detail below. For each paradigm, a drop assay was utilized to test *C. elegans* avoidance. The cooler color of the paradigm represents the naïve condition and the warmer color represents the pre-exposed condition.

*C. elegans* Selection and Maintenance

The N2 strain of *C. elegans*, obtained from the *Caenorhabditis* Genetics Center (CGC), was the original strain derived by Sydney Brenner. Brenner derived the N2 strain from a soil culture collected in Bristol, England by Ellsworth Dougherty in spring of 1964 (Riddle et al., 1988). This strain serves as the wild type strain for most behavioral and genetic baseline research performed with *C. elegans* and was therefore selected for use in this project. N2 *C. elegans* were maintained on 6 cm nematode growth media (NGM) plates seeded with OP50 *E. coli* to provide an adequate food source and were passed every few days to prevent starvation and overcrowding. N2 *C. elegans* were stored in the 20°C incubator throughout the course of the project.

Drop Assays

Drop assays were performed to compare the avoidance behaviors of pre-exposed and naïve *C. elegans*. An example of the drop assay can be seen below in Figure 3.

![Figure 3. Drop Assay](image)

*Figure 3. Drop Assay.* Shows response to neutral stimulus (top row) and response to a repellant (bottom row). A small drop of solution is placed at the tail of a decided animal, and drawn up to the head through
Capillary action. The animal either continues in the same direction (no response) or changes direction, making an “omega turn” (avoidance response). Adapted from Christopher Chute

*C. elegans* were washed from 6cm plates using M9 solution and transferred to a 1.5 mL Eppendorf tube utilizing a plastic transfer pipette. They were left to sit for 15 minutes then washed again with M9 and left to sit for another 15 minutes before being re-plated using a 100 μL micropipette onto 6cm plates without any bacterial food. *C. elegans* were allowed to sit for one hour in order to ensure plate drying and starvation. Drops of the solvent control (SC) of 1% ethanol were placed on 10 worms’ tails and enveloped the worm to reach the head and drawn up to the nose through capillary action. The avoidance behavior (avoid or not avoid) was recorded. Avoidance was defined as a change of more than 90° from the direction the worm was traveling in at the time the drop was administered. The Avoidance Index (AI) of a plate was calculated by dividing the number of worms that avoided by the total number of worms assayed. An example calculation is shown below:

\[
\text{AI test} = \frac{\text{Worms Avoided}}{\text{Total Worms Assayed}} = \frac{6}{10} = 0.6 \text{ or } 60\%
\]

This drop assay procedure was then completed again on the same 10 worms, utilizing 1μM osas#9 instead of 1% EtOH, and the AI for the aversive chemical was calculated.

**Transient Assay**

The pre-exposure for this assay design occurs through an initial drop assay performed on first larval (L1) stage *C. elegans*. Assay plates were made by obtaining 6cm NGM plates and seeding them with OP50 bacteria to provide a food source. One N2 hermaphrodite adult *C. elegans* was placed on each plate and allowed to lay eggs. The eggs were allowed to mature to the first larval (L1) stage in the 20°C incubator. The L1 worms utilized for the naïve condition were washed twice with M9 buffer and transferred to new 6cm NGM plates to mature to young adult (YA) stage, once again in the 20°C incubator. The L1 worms utilized for the pre-exposure condition were washed twice with M9 buffer, then a drop assay was performed on these L1 worms. Following the drop assay, the L1 worms in the pre-exposure condition were transferred to new 6cm NGM plates with OP50 bacteria added and allowed to mature to the YA stage in the 20°C incubator. When the worms for both the naïve and pre-exposure conditions had reached the YA stage, they were washed twice with M9 buffer and a drop assay was performed on each naïve and pre-exposure plate. Figure 4 shows a diagram describing this procedure.
Acute Multi-Generation Assay

The pre-exposure for this assay design occurs through acute exposure of YA *C. elegans* to osas#9 in an Eppendorf tube. 6cm NGM plates with OP50 bacteria and containing 20-50 young adult *C. elegans* were washed twice with M9 buffer and transferred to a 1.5 mL Eppendorf tube. The M9 buffer was removed and 100 μL of either 1 μM osas#9 (pre-exposed) or 100 μL of M9 (control) was added to the Eppendorf tubes containing the young adult *C. elegans*. The mixture was left to sit for 30 mins and then excess osas#9 or M9 was removed from the tube and the animals were washed twice with M9 buffer. From the Eppendorf tube, one young adult hermaphrodite was removed and placed on its own 6cm NGM plate with OP50 bacteria. The plates were placed in the 20°C incubator and left for 3 days for the hermaphrodite to lay eggs and have them hatch and grow up to young adult stage. A drop assay was then done on these young adult offspring. Figure 5 shows a diagram describing this procedure.
Pheromone and Control Added on top of Plates, Same-Generation Assay

The pre-exposure for this assay design occurs through acute exposure of C. elegans eggs to osas#9 present on top of the NGM plates. Two different plates were used: 6 cm control plates with 100 μL of 1% ethanol added on top of the OP50 bacteria or 6 cm pre-exposure plates with 100 μL of 1 μM osas#9 added on top of the OP50 bacteria. The liquid was left to sit and soak into the plates for 1 hour. To start the assay, one hermaphrodite C. elegans was added to each 1% ethanol and 1 μM osas#9 plate and left for 4-5 hours in the 20°C incubator for eggs to be laid. The hermaphrodites were removed from the plates and the plates were return to the 20°C incubator for 24 hours for the eggs to mature to L1 stage. The plates were then washed twice with M9 buffer and a syringe was utilized to transfer the remaining L1 worms to new 6cm NGM plates with just OP50 bacteria. These plates were left in the 20°C incubator for 2.5 days to allow the L1 worms to mature into young adults. A drop assay was then performed on each naïve plate and each pre-exposure plate. Figure 6 shows a diagram detailing this procedure.
Figure 6. Compound on plate method for testing memory of osas#9 avoidance in *C. elegans*. Naïve condition is shown on the left and pre-exposure is shown on the right. One hermaphrodite is placed on the plate to lay eggs and is then removed. Pre-exposure occurs from egg to L1 in absence of the mother.

Assay Plates with Pheromone and Control, Same-Generation Assay

The pre-exposure for this assay design occurs through acute exposure of *C. elegans* eggs to osas#9 present within the NGM plate itself. This assay design was inspired by a paper from Hong et al., where the authors exposed worms to an attractive ascaroside within the assay plates themselves and observed noticeable behavioral differences (Hong et al., 2017). Two types of assay plates were made for this assay design: 35mm control plates containing 1% ethanol and 35mm pre-exposure plates containing 1 μM osas#9. To make these plates, 1.25 mL of 100% ethanol and 1.25 mL of 100 μM osas#9 were added to respective flasks of 125 mL of NGM following autoclaving to prevent degradation of the chemicals. Once poured, the plates were stored at 4°C until they were needed for assaying. To start the assay, one hermaphrodite *C. elegans* was added to each 1% ethanol plate and each 1 μM osas#9 plate and left for 4-5 hours in the 20°C incubator for eggs to be laid. The hermaphrodites were removed from the plates and the plates were returned to the 20°C incubator for 24 hours for the eggs to mature to L1 stage. The plates were then washed twice with M9 buffer and a syringe was utilized to transfer the remaining L1 worms to new 6 cm NGM plates with just OP50 bacteria. These plates were left in the 20°C incubator for 2.5 days to allow the L1 worms to mature into young adults. A drop assay was then performed on each naïve plate and each pre-exposure plate. Figure 7 shows a diagram describing this procedure.
Assay Plates with Pheromone and Control, Multi-Generation Assay

This assay design tests the F₁, F₂, and F₃ generations, or the offspring of the *C. elegans* eggs which were pre-exposed to osas#9 present within the NGM plate itself. Prior to testing the P₀ worms, as described in the protocol above, one naïve and one pre-exposure hermaphrodite were moved to new 6 cm NGM plates with just OP50 bacteria and left for 4-5 hours in the 20°C incubator for eggs to be laid. The hermaphrodites were removed from the plates and the plates were returned to the 20°C incubator for 2.5 days to allow the eggs to mature into young adults; no washing step occurred at the L₁ stage. A drop assay was then performed on each naïve plate and each pre-exposure plate; prior to testing, the protocol was repeated to provide F₂ young adult worms for testing and then again to provide F₃ young adult worms for testing.

Assay Plates with Alternate Pheromone and Control, Same-Generation Assay

This assay design works to test that the paradigm for assessing an epigenetic memory of osas#9 is specific to osas#9 and not to all aversive chemicals. The pre-exposure for this assay design occurs through acute exposure of *C. elegans* eggs to an alternate aversive chemical not naturally produced, such as 500 mM glycerol, present within the NGM plate itself. Two types of assay plates were used for this assay design: 35mm normal NGM plates and 35mm pre-exposure plates containing 500 mM glycerol. To make the pre-exposure plates, 9.2 mL of 13.57 M glycerol was added to 250 mL of NGM following autoclaving to prevent degradation of the chemical. Once poured, the plates were stored at room temperature until they were needed for assaying. To start
the assay, one hermaphrodite *C. elegans* was added to each normal NGM and 500 mM glycerol plate and left for 4-5 hours in the 20°C incubator for eggs to be laid. The hermaphrodites were removed from the plates and the plates were returned to the 20°C incubator for 24 hours from the eggs to mature to L1 stage. The plates were then washed twice with M9 buffer and a syringe was utilized to transfer the remaining L1 worms to new 6 cm NGM plates with just OP50 bacteria. These plates were left in the 20°C incubator for 2.5 days to allow the L1 worms to mature into young adults. A drop assay was then performed on each naïve plate and each pre-exposure plate. Figure 8 shows a diagram describing this procedure.

**Figure 8. Paradigm for testing memory of 500 mM glycerol avoidance in C. elegans.** Naïve condition is shown on the left and pre-exposed is shown on the right. Color indicates control or chemical in the plates. One hermaphrodite was placed on each plate to lay eggs and then removed before eggs hatched and became L1s.

**Assay Plates with Alternate Pheromone and Control, Multi-Generation Assay**

This assay design tests the F1, F2, and F3 generations, or the offspring of the *C. elegans* eggs which were pre-exposed to 500 mM glycerol present within the NGM plate itself. Prior to testing the P0 worms, as described in the protocol above, one naïve and one pre-exposure hermaphrodite were moved to new 6cm NGM plates with just OP50 bacteria and left for 4-5 hours in the 20°C incubator for eggs to be laid. The hermaphrodites were removed from the plates and the plates were returned to the 20°C incubator for 2.5 days to allow the eggs to mature into young adults; no washing step occurred at the L1 stage. A drop assay was then performed on each naïve plate and each pre-exposure plate; prior to testing, the protocol was repeated to provide F2 young adult worms for testing and then again to provide F3 young adult worms for testing.
Statistics

Statistics and graphs were done using GraphPad Prism software. Data was analyzed using unpaired T tests and ordinary one-way ANOVAs with multiple comparisons. The specific statistics utilized for each method are outlined in the results and discussion section.
Results and Discussion:

Based on the previously described methodology, this section begins with the various paradigms tested, results obtained, and various improvements to each paradigm made, with explanations as to why. Next, the results and discussion move on to the finalized paradigm and avoidance data obtained for the parental generation and three familial generations for a biogenic produced stimulus, osas#9. It was observed that there is an epigenetic memory to osas#9, and we wanted to see if this memory would be specific to biogenic stimuli, or if another aversive stimulus, glycerol, would create the same epigenetic memory. The results section finishes by comparing the percent change of the osas#9 pre-exposure to the glycerol pre-exposure which helps strengthen the fact that there are epigenetic mechanism involved in the memory to osas#9 but not glycerol.

Developing the Paradigm for Epigenetic Study

Three different paradigms were tested before it was decided that the fourth and final paradigm was the most consistent and gave highly repeatable results. Each initial paradigm tested only the parental, or P0, generation. The fourth paradigm was moved on to test multiple filial, or Fx, generations. Additionally, the color coding remains the same throughout this section of the paper, blue is naïve and orange is pre-exposed to osas#9 with the lighter shade as solvent control avoidance and the darker shade as osas#9 avoidance.

Transient Pre-Exposure

The first method utilized for testing epigenetic memory to aversive chemicals was a transient pre-exposure method. For this method, C. elegans had a brief pre-exposure of a drop assay in the L1 life-stage to over expose them to osas#9. No data was obtained for the pre-exposure since the purpose of the pre-exposure was to over-expose the worms without recording their initial avoidance. Their avoidance was then tested and recorded at YA stage and can be seen below in Figure 9.
Figure 9. Transient Pre-Exposure Avoidance Index (AI) ± SEM for naïve and pre-exposed worms. Assay shows no difference between naïve and pre-exposed animals.

An unpaired t-test was performed on the data in Figure 9, which indicated no significant difference between naïve and pre-exposed avoidance of osas#9 utilizing this pre-exposure method. It was hypothesized that transient pre-exposure was not long enough to allow the L1 worms to be consistently exposed to osas#9, so there was no detectable memory of this pre-exposure when the same worms were tested as young adults. A longer, more consistent exposure was deemed necessary, and was therefore the next exposure method that was assayed.

Acute Pre-Exposure

The next exposure tested was an acute pre-exposure where the *C. elegans* were pre-exposed in an Eppendorf tube containing osas#9 for 30 mins. This pre-exposure was longer than the transient pre-exposure, so it was hypothesized that a difference in behavior between naïve and pre-exposed would be observed. The avoidance data for the acute pre-exposure can be seen in Figure 10.
Figure 10. Acute Pre-Exposure Avoidance Index (AI) ± SEM for naïve and pre-exposed worms. Top graph is for Emily and bottom graph is for Isabella. Trends in the data were inconsistent between the two experimenters, so individual graphs were shown. No change in avoidance was observed for one scientist and an increase was observed for the other scientist. * = p < 0.05

The data for this experiment was broken up to show Emily and Isabella’s data separately. Emily’s data is shown on the top half of Figure 10 and Isabella’s data is shown on the bottom half. The data was broken up like this because there were clear differences between Emily and Isabella’s pre-exposure and avoidance results. An unpaired t-test was run, and showed that for Emily there was no difference between the naïve and pre-exposed osas#9 avoidance, whereas Isabella had a significant increase in avoidance for pre-exposed versus naïve. When the data was averaged together, it showed neither a significant increase nor decrease in avoidance between naïve and pre-exposed. The combined avoidance data for the acute pre-exposure can be seen in Figure 11.
In order to understand why the discrepancies in results were occurring, both scientists completed their own pre-exposure, but then switched half of their plates. This allowed them to test half of their own pre-exposure and half of the others’. This method was used to see if there was a difference within the pre-exposure. No matter who pre-exposed the *C. elegans*, Emily saw no difference between naïve and pre-exposed and Isabella saw a decrease in avoidance, leading to the hypothesis that there was a difference in the drop assay between Emily and Isabella. Both scientists recorded a video when completing the drop assay, then viewed the videos together and compared scoring. From this it was determined that Emily was at times testing animals that were still indecisive, while Isabella consistently tested only worms that were moving in a straight line. With this error noted and corrected, more consistency was achieved in assay results between the two scientists.

Even with the drop assay methods fixed, there were still concerns with the acute pre-exposure assay. In the procedure, the worms were first washed in M9 and then most of the buffer was removed before adding the control or the treatments. However, it is impossible to remove all of the M9 without disturbing the small pellet of *C. elegans* in the Eppendorf tube. This meant that the osas#9 treatment was getting slightly diluted, but it was uncertain how diluted the treatment would be. Due to this error and inconsistency, it was deemed more research had to be done and a new method needed to be created.
Pre-Exposure on Plates

To allow for prolonged exposure at a younger life stage, the next method tested *C. elegans* which had been pre-exposed to the ascaroside on the surface of plates as eggs. These pre-exposed eggs were then washed and moved to normal plates in the L1 stage, after approximately 24 hours of pre-exposure, which allowed the worms to mature without the possibility of habituation to the ascaroside. Once they had matured to the YA stage, the worms were tested with the drop assay. Data from the parental generation for this method can be seen in Figure 12.

![Figure 12. Compound on Plate Avoidance Index (AI) ± SEM for naïve and pre-exposed worms.](image)

A significant decrease in avoidance between naïve and pre-exposed was observed, but there was concern that ethanol or osas#9 had not soaked into the whole plate, leading to uneven pre-exposure. ** = p < 0.01, *** = p < 0.001

When an unpaired t-test was performed, the data in Figure 12 indicated a slight significance between the solvent controls, but also a much stronger significance in avoidance of osas#9 between the naïve and pre-exposed conditions than had been noted in any of the previous exposure methods. There was a three star significant decrease in avoidance between naïve and pre-exposed animals within the parental generation, which was seen consistently for both scientists. However, there was the concern with this method that the ethanol for the naïve plates and the osas#9 for the pre-exposure plates had not fully soaked into the whole plate and could have evaporated, which would lead to uneven pre-exposure. There was also no guarantee that that the chemicals were evenly distributed over the plate, leading to uneven pre-exposure. It was deemed necessary to amend this method to allow for consistent pre-exposure and to prevent evaporation of the chemicals.
Pre-Exposure in Plates

The final procedure tested and eventually becoming our paradigm involved adding the ascaroside to the agar before the plates were poured. This ensured that the osas#9 was distributed evenly throughout the plate and would not evaporate off the top. *C. elegans* were grown from egg to L1 on these plates, pre-exposing them for about 24 hours. They were then washed and moved to normal NGM plates and allowed to grow up to YA where they were then tested with the drop assay. Data from the parental generation can be seen in Figure 13.

**Figure 13. Compound in Plate Avoidance Index (AI) ± SEM for naïve and pre-exposed worms.** This pre-exposure showed a significant decrease between naïve and pre-exposed osas#9 avoidance. 15 plates were tested for naïve and 15 plates were tested for pre-exposed resulting in 150 animals for each condition. ** = p < 0.01, **** = p < 0.0001

The data in Figure 13 shows that there is a significance between the solvent controls for naïve and pre-exposed *C. elegans*, but also a much stronger significance in avoidance of osas#9 between naïve and pre-exposed. There was a four star significant decrease in avoidance between naïve and pre-exposed animals when an unpaired t-test was performed, which was consistent between both scientists. Because of the strength of this decrease and the consistency between scientists, this method was deemed the most effective paradigm and used for all future testing.

Established Paradigm for Epigenetic Study

After noticing a consistent decrease and memory in the first generation (Figure 13), we wanted to see if this memory would be passed on to the following generations. *C. elegans* that underwent the pre-exposure or naïve condition, but weren’t tested with the drop assay, were moved on to lay eggs on fresh NGM plates. Again, after the eggs were laid the mother was removed and
the eggs were left to grow up to YA where they were tested in the drop assay. This was repeated for 3 filial generations after the parental and initial pre-exposure. The avoidance data for the parental (P0) and three filial (F1 to F3) can be seen in Figure 14. 15 plates of naïve C. elegans and 15 plates of pre-exposed C. elegans were assayed for all generations (P0 through F3).

Figure 14. Avoidance Index (AI) ± SEM for naïve and pre-exposed worms to 1 μM osas#9 across three filial generations. Data was obtained using paradigm of pre-exposure in plates. The first filial generation (F1) was obtained by moving along one YA to lay eggs and then removed before eggs hatch and become L1s. 15 plates were used for naïve and pre-exposed conditions across all generations, resulting in 150 animals for each condition in each generation. A significant decrease in avoidance to osas#9 between naïve and pre-exposed was observed across all three generation. A decrease in naïve avoidance to osas#9 was observed in the F1, F2, and F3 generations. ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 ■ signifies a p values of *** from P0 naïve 1 μM osas#9 and ★ signifies a p value of *** from P0 pre-exposed 1 μM osas#9.

Figure 14 shows that there is a statistically significant decrease in avoidance between the naïve and pre-exposed C. elegans in the parental generation that is observed up through three filial generations. For all generations, there is a lower avoidance to osas#9 after pre-exposure, indicating a sustained memory to osas#9 after pre-exposure. There is also an overall decrease in avoidance to osas#9 for naïve and pre-exposed C. elegans which is indicated by the squares and stars. One possible reason for this is that the animals are growing up in isolation and no longer have their mother present. The mother might be necessary to help relay information necessary to trigger and learn the avoidance response. Another possible explanation is that the animals are getting starved in the P0 generation. This starvation could somehow impact both naïve and pre-exposed animals leading to an overall decrease in avoidance to osas#9, but still there is a significantly lower avoidance after pre-exposure.
Graphs of the day-by-day data are included in Appendix 1 to show that there was consistency between experimenters and on multiple days. The consistency of multiple days and assays strengthens the evidence for an epigenetic memory observed in pre-exposure to osas#9.

**No Epigenetic Memory of Non-Native Aversive Stimuli**

With the successful establishment of a paradigm for epigenetic study, we wanted to determine if the decreased avoidance in the pre-exposed worms was specific to biogenic aversive stimuli such as osas#9 or if it was generally observed in response to all aversive stimuli. The color coding in this section is blue for naïve and red for pre-exposed to glycerol with the lighter shade as solvent control and darker shade as glycerol avoidance.

**Glycerol**

The chemical selected for comparison with osas#9 was glycerol. Glycerol is a known aversive chemical not naturally produced by *C. elegans* and not naturally found in the *C. elegans’* environment, making it a non-native aversive stimulus. Figure 15 indicates the chemical structure of glycerol.

![Glycerol structure](image)

**Figure 15. Glycerol structure.** Obtained from Fisher Scientific

Glycerol was added to the agar before the plates were poured, as with the osas#9 assays, to ensure even distribution of the chemical through the entire plate. 500 mM was the decided upon final glycerol concentration for the pre-exposure plates, as this concentration was shown to yield ⅔ avoidance response when tested which was consistent with the response to 1 μM osas#9. Normal agar plates were used for the naïve condition, as the agar already contained water which would serve as the solvent control for the drop assay. *C. elegans* were grown from egg to L1 on these plates, pre-exposing them for about 24 hours. They were then washed and moved to normal NGM plates and allowed to grow up to YA where they were then tested with the drop assay. The data for the parental generation can be seen below in Figure 16 and shows no significant difference between the naïve and pre-exposed conditions.

*C. elegans* that underwent the pre-exposure to glycerol or the naïve condition, but weren’t tested in the drop assay, were moved on to lay eggs on fresh NGM plates. Again, after the eggs
were laid the mother was removed and the eggs were left to grow up to YA where they were tested with the drop assay. This was repeated for 3 filial generations after the parental and initial pre-exposure. The avoidance data for the three filial (F$_1$ to F$_3$) can be also be seen in Figure 16.

![Avoidance Index ± SEM for naïve and pre-exposed worms to 500 mM glycerol across three filial generations.](image)

Figure 16. Avoidance Index (AI) ± SEM for naïve and pre-exposed worms to 500 mM glycerol across three filial generations. The same procedure used for multi-generational osas#9 pre-exposure was used for glycerol. No significant difference between naïve and pre-exposed avoidance to 500 mM glycerol ■ signifies a p values of *** from P$_0$ naïve 1 μM osas#9 and ★ signifies a p value of * from P$_0$ pre-exposed 1 μM osas#9.

Figure 16 shows that there is no significant difference in avoidance of 500 mM glycerol between the naïve and pre-exposed conditions for any of the three filial generations. As was seen with the osas#9 data detailed in Figure 15 above, there was an overall decrease in avoidance of glycerol for naïve and pre-exposed *C. elegans* which is indicated by the squares and stars. Again, we hypothesize that this overall decrease noted could be due to the animals growing up in isolation from their mothers or could be due to their starvation in the P$_0$ generation.

Graphs of the day by data are included in Appendix 2 to show that there was consistency between experimenters and across multiple days. The consistency of multiple days strengthens the fact that there is no epigenetic memory to glycerol from one generation to the next on multiple days by multiple experimenters.

Comparing osas#9 and glycerol

To prove that there was indeed an epigenetic memory to osas#9, but not to glycerol, a percent change of avoidance was calculated for all generations in both treatments. The data was first normalized taking the log$_{10}$ of each value and then a fold change between aversive stimuli avoidance and the solvent control avoidance was calculated. This was achieved by dividing the
normalized avoidance to the aversive stimuli by the normalized avoidance to the solvent control. A percent change was then calculated by subtracting the naïve fold change from the pre-exposed fold change, dividing by the absolute value of the naïve fold change, and then multiplying by 100 to get a percentage. A sample calculation is shown below:

\[
\text{Percent Change} = \frac{\text{Pre exposed Fold Change} - \text{Naïve Fold Change}}{|\text{Naïve Fold Change}|} \times 100
\]

The percent change of osas#9 pre-exposed and glycerol pre-exposed data is shown in Figure 17.

![Graph showing percent change for normalized osas#9 and glycerol fold-change data. A consistent decrease was observed for naïve vs pre-exposed osas#9 animals around 35% indicated by the dashed line. The naïve vs pre-exposed 500 mM glycerol animals did not have as large of a percent change as osas#9 pre-exposed animals.](image)

**Figure 17. Percent change for normalized osas#9 and glycerol fold-change data.** A consistent decrease was observed for naïve vs pre-exposed osas#9 animals around 35% indicated by the dashed line. The naïve vs pre-exposed 500 mM glycerol animals did not have as large of a percent change as osas#9 pre-exposed animals.

Figure 17 shows a consistent percent change of around -35% for osas#9 exposed *C. elegans* whereas glycerol pre-exposed animals have an inconsistent change of approximately +/- 5%. The percent change for osas#9 stays strong and even begins to increasingly decrease from the F1 to the F3 generation, meaning the memory is consistent throughout. While the same procedure was carried out by pre-exposing *C. elegans* to glycerol, a non-native aversive stimulus, the same trend was not noted. This data is very indicative that there is an epigenetic memory to osas#9 after the initial pre-exposure whereas there is no memory to glycerol.
Conclusions and Future Studies:

From our work throughout this project, we have reached two major conclusions. First, we were able to develop a paradigm for consistently testing behavioral epigenetics in *C. elegans* with use of ascarosides. There was a lot of trial and error, as well as reworking of methodology, that was diligently completed before a successful paradigm could be established. Both scientists applied a lot of time and effort into standardizing the paradigm to ensure it would be easily repeatable by future scientists. Our hope is that this project will open new pathways of study within the laboratory with focus on behavioral epigenetics and its implications.

Second, we determined that there is an epigenetic memory to biogenic produced aversive stimuli but not to non-native aversive stimuli. The pre-exposure to osas\#9 created a decrease in avoidance between pre-exposed and naïve animals of about 35%. This decrease was consistent in the parental generation all the way up through the F3 generation, and even appeared to get stronger throughout the three filial generations tested. There was no difference between the naïve and pre-exposed avoidance values for the glycerol procedure or the percent change, indicating that the observed effect is not olfactory imprinting and that there is a memory to biogenic produced stimuli but not non-native aversive stimuli.

The bigger implications of this project relate to survival and the innate behaviors of *C. elegans*. *C. elegans* would never see an increased amount of osas\#9 in their environment, unless they were starved. During this starvation, they appear to have a memory to osas\#9 which can be passed on to their offspring. This memory makes it so they avoid osas\#9 less and hence would be more likely to stay in the area without food. From this behavior we can gather that starved animals will be less likely to leave their low food environment, resulting in a lower chance of survival. This indicates the process of evolution, with natural selection acting upon those animals with a greater chance of survival.

We have identified several ways in which our project could be continued. First, it would be important to determine how many generations the epigenetic memory to osas\#9 is carried out. Within the scope of our project, we determined that the memory of avoidance to osas\#9 following pre-exposure was sustained across three filial generations, or through to at least the great-grandchildren of the pre-exposed animals. Testing from the fourth filial generation onward could allow determination of how many more generations the memory is passed along. It would also be
interesting to see if the percent change in decreased avoidance continues to get stronger as the generations progress.

Another important continuation of our project would be to determine what epigenetic mechanisms are involved, as well as which genes or histones are being modified. The results of our assays and the consistent percent change of -35% in our pre-exposed animals provides strong evidence that the pattern we are observing is not simply olfactory imprinting, but rather a sustained memory transmitted across multiple generations. In order to determine the epigenetic underpinning of this memory, it would be necessary to determine what genes are implicated in the response to osas#9; with these genes identified, the next step would be to examine changes in methylation or acetylation of these genes between normal and pre-exposed animals. Any changes in methylation or acetylation could help explain the epigenetic mechanisms at work in the memory of decreased avoidance to biogenic produced stimuli following pre-exposure.

An additional project we would recommended would be to complete the same paradigm without having the young C. elegans grow up in isolation. For this project, mother should be kept on the plate instead of being removed to see if the epigenetic memory and decreased avoidance is still consistent, or if it decreases in strength, or disappears completely. This project would help us gain a better understanding of the significant decrease in avoidance from the parental generation, noted in all subsequent generations for both the naïve and pre-exposed conditions. As was mentioned before, the presence of the mother might be necessary to help relay information necessary to trigger and learn the avoidance response; this suggested project would allow for determination of the validity of this hypothesis.
References:


*Discover.*


10.1126/science.282.5396.2012 Retrieved from
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Appendix

Appendix 1. Day by day graph for osas#9 pre-exposure

* P < 0.05

Appendix 2. Day by day graph for glycerol pre-exposure