

Video Article

Measuring the Effects of Bacteria on *C. Elegans* Behavior Using an Egg Retention Assay

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Abstract

C. elegans egg-laying behavior is affected by environmental cues such as osmolarity¹ and vibration². In the total absence of food *C. elegans* also cease egg-laying and retain fertilized eggs in their uterus³. However, the effect of different sources of food, especially pathogenic bacteria and particularly *Enterococcus faecalis*, on egg-laying behavior is not well characterized. The egg-in-worm (EIW) assay is a useful tool to quantify the effects of different types of bacteria, in this case *E. faecalis*, on egg-laying behavior.

EIW assays involve counting the number of eggs retained in the uterus of *C. elegans*⁴. The EIW assay involves bleaching staged, gravid adult *C. elegans* to remove the cuticle and separate the retained eggs from the animal. Prior to bleaching, worms are exposed to bacteria (or any type of environmental cue) for a fixed period of time. After bleaching, one is very easily able to count the number of eggs retained inside the uterus of the worms. In this assay, a quantifiable increase in egg retention after *E. faecalis* exposure can be easily measured. The EIW assay is a behavioral assay that may be used to screen for potentially pathogenic bacteria or the presence of environmental toxins. In addition, the EIW assay may be a tool to screen for drugs that affect neurotransmitter signaling since egg-laying behavior is modulated by neurotransmitters such as serotonin and acetylcholine⁵⁻⁹.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51203/>

Introduction

Caenorhabditis elegans, a microscopic, free-living roundworm, is a model organism traditionally used to study developmental and cell signaling processes because of its transparent anatomy, well-characterized development, fully-sequenced genome, short generation-time, and genetic homology to humans. More recently, *C. elegans* has become a model organism in the field of environmental toxicology and innate immunity^{10, 11}.

These self-fertilizing hermaphroditic worms become sexually mature within two to three days of hatching from the egg. During its life cycle, *C. elegans* passes through four larval stages (L1-L4), before reaching adulthood. One isolated hermaphrodite can produce, on average, 300 offspring within three days of peak fecundity. In reproductively mature *C. elegans* hermaphrodites, fertilized eggs are retained within the uterus for several hours before being laid. The normal number of eggs stored in the uterus at any one time (during peak fecundity) is between ten and fifteen¹². The number of eggs in the uterus is a function of both the rate of egg production and the rate of egg laying. Fertilized eggs are expelled from the uterus by the contraction of sixteen vulval muscles arranged around the opening of the vulva¹³. Hermaphrodite-specific motoneurons (HSN's) and VC motoneurons synapse onto vulval muscles affecting muscle contraction and thus egg-laying behavior^{5,7,13,14}. Expulsion of eggs from the uterus occurs due to coordinated activity of neurons and muscles.

Lab cultures of *C. elegans* are typically raised on a diet of nonpathogenic *Escherichia coli* OP50. In the natural environment, *C. elegans* come into contact with a variety of food sources, such as pathogenic bacteria, that can be potentially harmful. When exposed to harmful substances in the environment, *C. elegans* retain eggs until the environment becomes more favorable. Presumably this egg retention is an effort to protect their progeny.

In this egg in worm (EIW) assay, *C. elegans* are exposed to the potentially pathogenic bacteria, *Enterococcus faecalis*, which is found in the environment. Exposure to pathogenic forms of *E. faecalis* can cause persistent intestinal infection and even death in *C. elegans*¹⁵. Exposure to other forms of pathogenic bacteria have been shown to affect egg retention^{16,17}, however the effect was not quantified. In addition, the effect of mildly pathogenic strains of *E. faecalis*, strains that are not immediately lethal, on egg-laying behavior has not been studied.

EIW assays involve counting the number of eggs retained in the uterus of *C. elegans*⁴. Even though *C. elegans* are transparent, eggs accumulating in the uterus can be difficult to quantify in an intact animal. The EIW assay involves bleaching gravid adult *C. elegans* that were exposed to bacteria for a fixed period of time. The bleach solution dissolves the outer cuticle leaving the eggs behind. The eggs are refractive to

the effects of bleach due to the presence of a protective eggshell. After bleaching, one is very easily able to count the number of eggs released from the uterus of the worms upon bleaching.

The assay described is a simple, inexpensive, and quick method to quantify the number of eggs in the uterus at one time, and thus quantify the effects of *E. faecalis* on egg retention. This assay may be used to quantify the effect of other types of bacteria, environmental toxins or drugs on egg retention. This assay also has the potential to be used as a screen for bacterial pathogenicity.

Protocol

1) Preparation of Nematode Growth Media (NGM)

1. To make 50 plates, add 1.5 g NaCl, 8.5 g Ultrapure Agar, and 1.25 g peptone to a 1 L flask.
2. Add 487.5 ml of dH₂O to flask. Swirl gently to mix and cover opening of flask with a piece of aluminum foil.
3. Sterilize the solution by autoclaving at standard conditions (121 psi, 120 °C, 20 min).
4. Allow the solution to cool to 45 °C in a water bath.
5. To the solution, add the following in order: 500 µl cholesterol from a 5 mg/ml stock solution (dissolved in ethanol), 500 µl 1 M CaCl₂, 500 µl 1 M MgSO₄, and 12.5 ml KPO₄ buffer (54.15 g KH₂PO₄ and 17.8 g K₂HPO₄ in 500 ml of dH₂O) as described in¹⁸.
6. Using sterile pipettes, add 10 ml of agar solution to each 60 mm polystyrene Petri dish. Allow the media to solidify at room temperature overnight.

2) Preparation of B Broth *E. coli* Media

1. To make five 100 ml cultures, add 5.0 g of Bacto Tryptone, 2.5 g yeast extract, 5.0 g NaCl to a beaker.
2. Add sterile, distilled water to a final volume of 500 ml. Heat the solution on a hot plate, while stirring, until the solutes have dissolved.
3. Pour 100 ml of B broth into five glass bottles (at least 200 ml volume).
4. Sterilize the solutions by autoclaving at standard conditions (121 psi, 120 °C, 20 min).
5. Allow B broth to cool to room temperature before use.

3) Seeding *C. elegans* Maintenance Plates

1. After allowing NGM plates to dry at room temperature for at least one week, inoculate one 100 ml B broth culture with a few colonies of *E. coli* (OP50).
2. Grow *E. coli* culture at 37 °C overnight.
3. Pipette one-two drops (approximately 100 µl) of OP50 culture onto the center of each NGM plate. Plates are typically stacked lid-side up. When pipetting (seeding), begin by pipetting the culture onto the plate at the bottom of the stack. Work your way up each stack. Try not to move the plates after seeding, because it is important that the culture not get too close to the edges of the plate.
4. Allow the seeded plates to dry at room temperature for at least one week before using.
5. Seeded plates are stored (inverted) in sealed plastic containers at room temperature.

4) Maintaining *C. elegans* Strains

1. To maintain well-fed *C. elegans* stocks, use a worm pick to move three L4s or gravid adults to a new, seeded NGM plate every 3-4 days.
2. Store plates (inverted) preferably inside an incubator at 15- 22 °C. Cultures in the assays described were stored at 20 °C. *C. elegans* development is temperature-dependent. As maintenance temperature increases, time between developmental stages decreases.

5) Preparation of Tryptic Soy Agar (TSA) *E. faecalis* Culture Media

1. To make 50 plates, measure 500 ml of sterile, distilled water into a flask and bring to a boil, while stirring, on a hotplate.
2. Add 20 g of powdered TSA agar to flask and allow solution to dissolve.
3. Sterilize the solutions by autoclaving at standard conditions (121 psi, 120 °C, 20 min).
4. Allow the solution to cool to 45 °C in a water bath.
5. Using sterile pipettes, add 10 ml of agar solution to each 60 mm polystyrene Petri dish. Allow the media to solidify at room temperature overnight.

6) Preparation of Tryptic Soy Broth (TSB) *E. faecalis* Culture Media

1. To make 250 culture tubes, measure 500 ml of sterile, distilled water into a flask and warm on a hotplate while stirring.
2. Add 15 g of TSB agar powder to the flask and allow the powder to dissolve.
3. Sterilize the solutions by autoclaving at standard conditions (121 psi, 120 °C, 20 min).
4. Allow solution to cool to room temperature, then pipette 2 ml into sterile, 5 ml test tubes.

7) Preparation of Brain Heart Infusion (BHI) Media, with Streptomycin, for *E. faecalis* Culture

1. Measure 500 ml of sterile, distilled water into a flask and bring to a boil, while stirring, on a hotplate.
2. Add 26 g of BHI medium to the flask and allow the solution to dissolve.
3. Sterilize the solutions by autoclaving at standard conditions (121 psi, 120 °C, 20 min).
4. Allow solution to cool to 45 °C in a water bath.
5. Using sterile technique, add 0.5 ml of a 10 mg/ml streptomycin stock solution and swirl to mix.
6. Add 10 ml of the solution to each 60 mm polystyrene Petri dish.
7. Allow media to solidify at room temperature overnight. Store plates (inverted) at 4 °C until a few hours before use.

8) Maintaining *E. faecalis* Strains

1. To maintain *E. faecalis* stocks, use a sterile loop to streak individual colonies onto separate tryptic soy agar (TSA) plates.
2. Grow cultures at 37 °C overnight. Store plates (inverted) in a sealed bag or box at room temperature for several weeks.

9) Preparing *E. faecalis* Plates for EIW Assay

1. Inoculate a 2 ml TSB culture with a colony of *E. faecalis* and incubate overnight at 37 °C.
2. Pipette 20 µl of *E. faecalis* culture onto the center of a prepared BHI-streptomycin plate, and incubate overnight at 37 °C.

10) Preparing *E. coli* Plates for EIW Assay (Control Plates)

1. Inoculate a 100 ml B-broth culture with a colony of *E. coli* OP50 and incubate overnight at 37 °C
2. Pipette 20 µl of the OP50 culture onto the center of an unseeded NGM plate and allow the seeded plates to dry at room temperature overnight.

11) Egg in Worm Assay

1. Pick 15- 20 staged-L4 *C. elegans* (**Figure 1**) onto the center of a lawn of bacteria on a prepared BHI-Strep-*E. faecalis* plate. Be careful not to transfer a lot of OP50 to the BHI plate. Pick the same number of L4s to a control NGM-OP50 plate.
2. Incubate plates for 40 hr at 20 °C.
3. Prepare 20% bleach solution. Mix a commercial bleach (6.0% sodium hypochlorite) with the appropriate volume of distilled water.
4. Add 10 µl drops of bleach solution to ten distinct locations on a plastic lid (of a worm or bacterial plate).
5. Using a worm pick, transfer one worm into each bleach drop. Gently swirl the tip of the pick in the bleach droplet to make sure the worm has washed off the end of the pick (confirm by viewing the droplet under a dissecting microscope).
6. Allow the cuticle to dissolve for approximately 10 min or until the worms burst open, expelling the eggs (**Figure 2**). Be careful not to transfer eggs from the plate.
7. Using a dissecting microscope, count the eggs in each drop of bleach.

Representative Results

This assay allows one to quantify the number of retained eggs within *C. elegans* after exposure to *E. faecalis*. L4 staged worms (characterized by transparent open space above their vulva, **Figure 1**) were exposed to *E. faecalis* through adulthood. After forty hours of exposure to *E. faecalis*, bleaching was performed. As the cuticle disintegrated in the bleach droplet, the eggs became more apparent (**Figure 2**). The number of retained eggs was easily quantified by counting while viewing under dissecting microscope.

More eggs were retained in the presence of *E. faecalis* strains tested, as compared to *E. coli* OP50 controls (**Figure 3**). There is some variation in the effect of any one strain of *E. faecalis* on individual worms, as seen from the error bars. However, the effect of strains of *E. faecalis* on egg retention within a population of worms does vary significantly from the controls. The degree of egg retention elicited by different strains of *E. faecalis* tested may also vary. This varied effect may be due to the expression of different virulence factors in each strain.

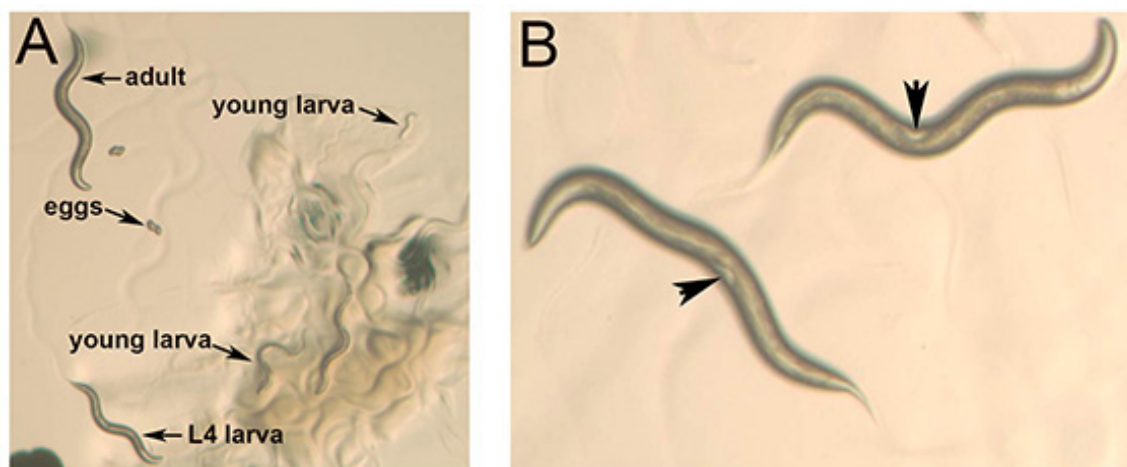


Figure 1. Identifying L4 staged *C. elegans*. **A.** L4 stage *C. elegans* larvae are the largest larvae present on plates- only slightly shorter in length than adult worms. L4s are thinner than adults as well, due to the absence of retained eggs (which make adult worms slightly wider). **B.** After identifying those larvae that are slightly shorter than adults, L4-staged *C. elegans* are further identified by the lighter half-moon spot located in the middle of their body (black arrowheads). This spot is visible in smaller larvae (L2 and L3 stages), but is absent in the adult. All images were taken of worms on NGM plates, with a camera mounted to a dissecting microscope (40X and 50X magnification for **A** and **B**, respectively). [Click here to view larger image.](#)

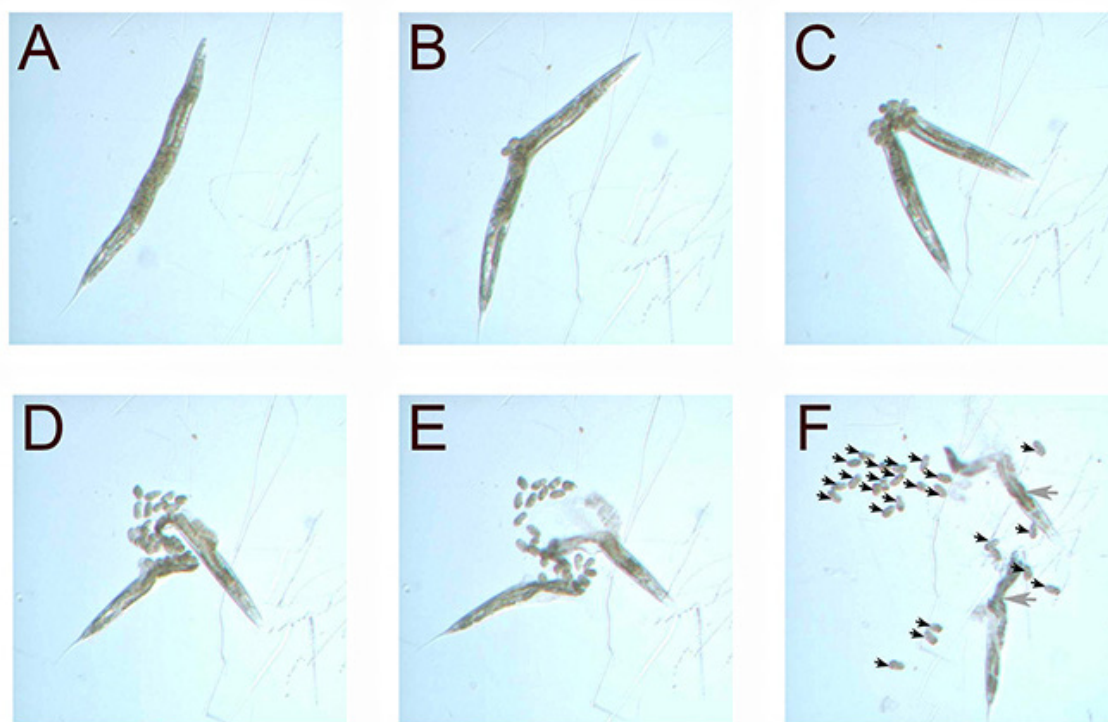


Figure 2. Bleach solution dissolves the *C. elegans* cuticle over time. A staged adult worm is placed in a droplet of bleach solution (**A**). Over five to ten minutes, the cuticle of the worm begins to dissolve (**B-F**). As most of the cuticle is dissolved, the eggs become visible (**F** black arrows). Fat stores inside the worm also dissolve slowly in bleach, and remain visible (**F**, gray arrows). All images were taken of worms on NGM plates, with a camera mounted to a dissecting microscope (40X). [Click here to view larger image.](#)

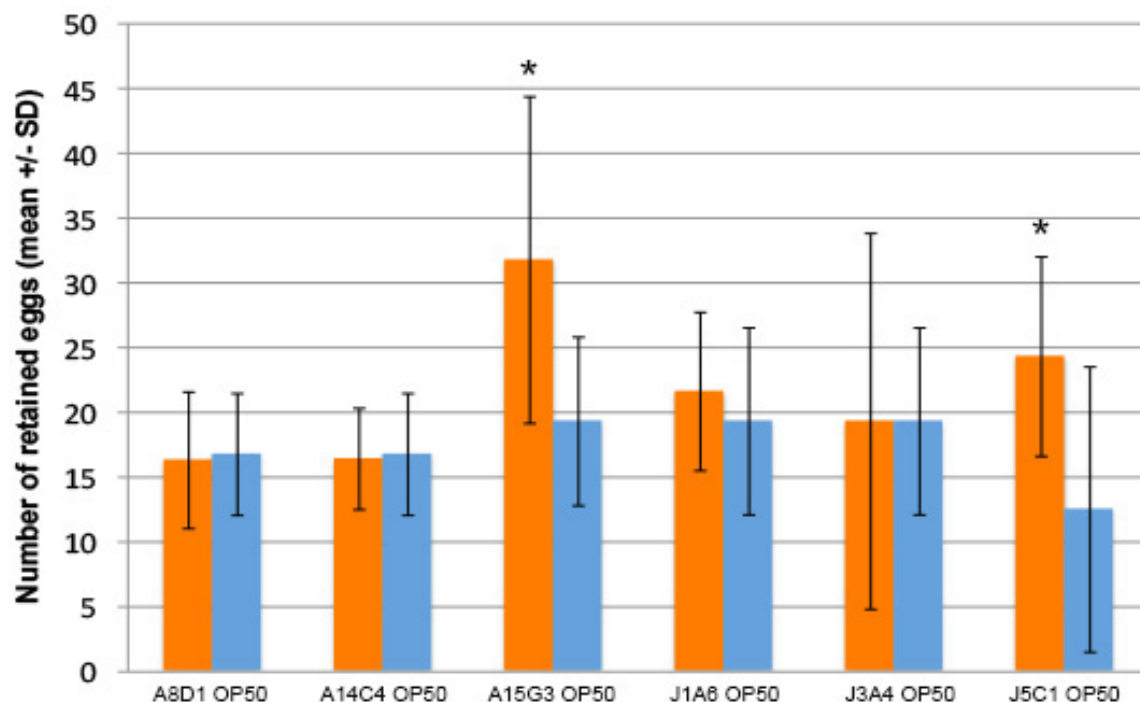


Figure 3. Some *E. faecalis* strains cause increased egg retention after 40 hours of exposure. Worms exposed to two of the six *E. faecalis* strains (orange bars) showed an increased level of egg retention compared to worms fed the *E. coli* (OP50) control strain (blue bars). Ten worms were fed each bacterial strain in each trial. Trials were done in triplicate on different days. * indicates $p < 0.05$ (Student's t-test). [Click here to view larger image.](#)

Discussion

The most critical steps in successfully performing this assay are: 1) using well-fed stocks of *C. elegans*, 2) culturing single types of bacteria on the assay plate, 3) accurately identifying staged L4 worms for exposure to *E. faecalis*, 4) keeping exposure time to *E. faecalis* consistent across all trials and 5) bleaching time should not exceed ten minutes to prevent egg disintegration.

For this assay it is important to pick healthy, well-fed worms. Maternal starvation can affect fecundity and growth of progeny^{19,20}, therefore it is important that worms have been well fed for several generations before performing this assay. A well-fed stock of worms is easily maintained by simply transferring a few adult worms to a new seeded NGM plate every 2-3 days. Two days before the assay, adult worms should be placed on a fresh lawn of OP50 in order to have sufficient L4 progeny available for the assay.

It is also important that EIW assay plates promote the growth of only a single type of bacteria. *E. faecalis* strains were grown on BHI plates. Streptomycin was added to the plates to select against *E. coli* OP50, which is transferred with the L4 worms as they are transferred from maintenance NGM plates to the assay plates. OP50 also grows on BHI plates. If not selected against, *C. elegans* would feed on two types of bacteria (*E. coli* and *E. faecalis*) while on the BHI plates. The strains of *E. faecalis* used in this assay are resistant to streptomycin. If different types of bacteria were used for this assay, a different antibiotic, and perhaps different culture media, should be used to select for the bacteria of choice and against *E. coli* OP50.

Accurately selecting staged worms for this assay is crucial for several reasons. First, it is important to count egg retention during the time of egg production/laying in the self-fertilizing *C. elegans*. Egg production occurs only during the first five days of *C. elegans* adulthood (peaking around 40 hours post-L4) and quickly declines thereafter^{4,21}. Adult worms live for an average of two to three weeks, so many adult worms live for at least a week while not producing fertilized eggs. Therefore, it is important to make sure that the EIW assay is not performed on unstaged adult worms of unknown age.

The second reason it is important to use staged animals in the EIW assay is so that the time in development at which worms have been exposed to the bacteria, toxin or drug is tightly controlled. Cultures of *C. elegans* are typically synchronized at the egg, L1 or L4 stages. The L4 stage was used for this assay because of the concern that longer exposure to *E. faecalis* would result in lethality before worms became gravid adults.

The duration of time worms are left in the bleach solution is also important to monitor. Worms should typically dissolve in under ten minutes, although this time does vary from worm to worm. Fertilized eggs are eventually dissolved by bleach, but the process is slower than for the cuticle due to the presence of a protective eggshell. The eggshell will begin to be dissolved by the bleach if left in the solution for longer than 10-15 min.

Egg retention reflects the balance between egg production and egg laying. The EIW assay alone does not distinguish whether the number of eggs retained in the uterus is due to alterations in egg production or egg laying. This is especially a concern if worms treated with a bacterial strain or toxin retain fewer eggs than those in the control group. In these cases especially, a follow-up brood size assay should be done. Brood size assays quantify the number of eggs laid over the worm's reproductive lifetime⁴. If the brood size of worms in the experimental and control groups are the same, then differences in egg retention can be attributed to differences in egg-laying behavior. Because it is unlikely that mildly

pathogenic bacterial strains like *E. faecalis* would act to increase egg production, it is reasonable to assume that an increase in egg retention after exposure to *E. faecalis* (as seen in **Figure 3**) is a result of decreased egg-laying behavior.

The EIW assay also does not determine the mechanism by which bacteria may alter egg retention. It is possible that *C. elegans* may retain eggs because the pathogenic bacteria affects feeding by colonizing in and blocking the mouth opening or because it is a poor food source. It is also possible that the bacteria may colonize and block the vulval opening, or affect the function of cells in the egg-laying system. Further analysis is required to determine the mechanism by which egg retention is altered.

This EIW assay can be easily modified to determine the effects of many types of compounds on egg retention. In addition, this assay is simple enough that it could be incorporated into a screen to identify genes, in either the host (*C. elegans*) or pathogen (*E. faecalis*), required for the effect of the pathogen. The EIW assay can also be used to screen for genes required for regulating egg-laying itself. *C. elegans* egg-laying behavior is modulated by neurotransmitters such as serotonin and acetylcholine⁵⁻⁹, and requires the coordinated activity of several neurons and muscle groups¹². EIW assays have been and continue to be used to identify genes important for neurotransmitter signaling and/or muscle activity. EIW assays provide a quantitative, rather than qualitative, analysis of an important *C. elegans* behavior.

Disclosures

The authors declare that they have no competing financial interests.

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References

- Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E., and Evans, P.D. Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science*. **216**, 1012-1014 (1982).
- Sawin, E.R. Genetic and cellular analysis of modulated behaviors in *Caenorhabditis elegans*. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA. (1996).
- Trent, C. Genetic and behavioral studies of the egg-laying system of *Caenorhabditis elegans*. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA. (1982).
- Chase, D.L., and Koelle, M.R. Genetic analysis of RGS protein function in *Caenorhabditis elegans*. *Methods Enzymol.* **389**, 305-320 (2004).
- Trent, C., Tsuing, N., and Horvitz, H.R. Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. *Genetics*. **104**, 619-647 (1983).
- Weinshenker, D., Garriga, G., and Thomas, J.H. Genetic and pharmacological analysis of neurotransmitters controlling egg laying in *Caenorhabditis elegans*. *J. Neurosci.* **15**, 6975-6985 (1995).
- Waggoner, L.E., Zhou, G.T., Schafer, R.W., and Schafer, W.R. Control of alternative behavioral states by serotonin in *Caenorhabditis elegans*. *Neuron*. **21**, 203-214 (1998).
- Bany, A., Dong, M., and Koelle, M.R. Genetic and cellular basis for acetylcholine inhibition of *Caenorhabditis elegans* egg-laying behavior. *J. Neurosci.* **23**, 8060-8069 (2003).
- Dempsey, C.M., Mackenzie, S.M., Gargus, A., Blanco, G., and Sze, J.Y. Serotonin (5HT), fluoxetine, imipramine and dopamine target distinct 5HT receptor signaling to modulate *Caenorhabditis elegans* egg-laying behavior. *Genetics*. **169**, 1425-1436 (2005).
- Leung, M.C., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., and Meyer, J.N. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. *Toxicol. Sci.* **106**, 5-28 (2008).
- Mylonakis, E. and Aballay, A. Worms and flies as genetically tractable animal models to study host-pathogen interactions. *Infect. Immun.* **73**, 3833-3841 (2005).
- Schafer, W. R. Egg-laying, *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.38.1. (2005).
- White, J., Southgate, E., Thomson, N. and Brenner, S. The structure of the *Caenorhabditis elegans* nervous system. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **314**, 1-340 (1986).
- Desai, C., Garriga, G., McIntire, S. L., Horvitz, H.R. A genetic pathway for the development of the *Caenorhabditis elegans* HSN motor neurons. *Nature*. **336**, 638-646 (1988).
- Garsin D.A., Sifri, C.D., Mylonakis, E. Qin, X., Singh, K.V., Murray, B. E., Calderwood, S.B., and Ausubel, F.M. A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10892-10897 (2001).
- Aballay, A., Yorgey, P. and Ausubel, F.A. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr. Biol.* **10**, 1539-1542 (2000).
- O'Quinn, A.L., Wiegand, E.M., and Jeddell, J.A. *Burkholderia pseudomallei* kills the nematode *Caenorhabditis elegans* using an endotoxin-mediated paralysis. *Cell. Microbiol.* **3**, 381-393 (2001).
- Brenner, S. Genetics of *Caenorhabditis elegans*. *Genetics*. **77**, 71-94 (1974).
- Harvey, S.C., Shorto, A. and Orbidans, H.E. Quantitative genetic analysis of life- history traits of *Caenorhabditis elegans* in stressful environments. *BMC Evol. Biol.* **8**, 15 (2008).
- Harvey, S.C. and Orbidans, H.E. All eggs are not equal: the maternal environment affects progeny reproduction and developmental fate in *Caenorhabditis elegans*. *PLoS One*. **6**, e25840 (2011).
- Klass MR. Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mech. Ageing. Dev.* **6**, 413-429 (1977).