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Choosy Worms to Teach Experimental Design

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In this module students learn core concepts in experimental design, data analysis and the 'messiness' of biological research by designing and analyzing their own research project using *C. elegans*, various chemicals, potential bacterial food sources or pathogens, and a simple behavioral assay. Participants in this workshop will design and carry out their own experiment and discuss the potential learning gains for students. This module can easily be adapted for a single period or a multi-week module and can be used in intro classes for majors/non-majors or ecology labs.

Keywords: CURE, Inquiry-based lab, *C. elegans*, experimental design

Introduction

There is an increasing call for undergraduate-level lab courses to provide authentic laboratory research experiences for introductory students. However, as many undergraduate courses, particularly at the introductory level, have high enrollments it is challenging to provide students with research experiences that truly give them the freedom to design their own research questions and experimental designs while constricting the materials prep work and necessary expertise of the instructors. Faced with this dilemma, Emory University developed a new module for our introductory biology lab course that allows students to work within the same basic experimental system with a small set of available reagents while still allowing students to develop their own research question and experimental approach.

In the Choosy Worm lab module students use behavioral assays with *Caenorhabditis elegans* (*C. elegans*) to address their own research questions usually in the areas of pathogen avoidance, food preference, as well as the genetics behind these behaviors. This lab challenges students to act as true scientists throughout their project from developing a research question, designing properly controlled experiments, to technically carrying out challenging experiments and properly analyzing their inherently messy data. One beneficial aspect of this module is that it uses an assay that is intuitive for students where they actually 'see' their results of worms moving towards/away from a stimulus. Thus, while these labs challenge students on all levels of scientific research skills, they do not require in-depth knowledge of molecular biology, allowing lab instructors to focus entirely on the process of biological research.

We currently use the Choosy Worm lab module over a period of 5 3-hour labs to give students a complete authentic research experience. Our implementation of the module also requires students to write a scientific research paper on their work. However, the lab module can easily be adapted to provide more guidance for any aspect/s of the research process to allow instructors to use fewer lab periods and focus on particular components of the research process. For example, it could be used in a single 2- or 3-hour lab period where the research question and experimental methods were provided to teach statistical analyses of messy data. For more in-depth projects it is useful to allow students to come in outside of normal lab hours to collect longer time-point data. Preparation time is generally minimal for the plates, chemicals and bacteria as these all can quickly and easily be prepared in bulk and stored but preparation of the *C. elegans* strains themselves must be planned in advance to ensure adequate time in order to grow up the amount needed.

Student Outline

These documents are provided to our students before their first lab period rather than as handouts during lab. Included documents should reflect the level of authentic research required of the students. Thus, the information on research questions and references could be omitted if the lab were to provide a question for the students while a document on designing the experiment could be included to guide the students through their experimental set-up to focus the module on other aspects of research.

Introduction to *C. elegans*

Caenorhabditis elegans is a species of roundworm that normally lives in the soil, and has been used in biology since the 1960s as an important model system. Its small size (~ 1 mm), short generation time (~3 days), and easy maintenance in the lab have allowed for a wealth of insights into development, genetics, neurobiology and behavior. Adult *C. elegans* contain a total of 959 cells and occur either as hermaphrodites or males.

Worms feed on bacteria, and in their natural environment they use chemical cues to locate their food. Worms can use both water-soluble and volatile chemicals to locate food; water-soluble chemicals are believed to help worms locate food in their nearby environment, whereas volatile chemicals are believed to help them locate food in areas that are farther away. Although some chemicals are attractive to worms (and allow them to find food), there are also many chemicals that are repulsive to worms (for example, because they are harmful).

Worms in the lab are typically raised in Petri dishes with agar and some bacteria. Although worms are quite a bit bigger than bacteria, they are still small to handle. Dissecting microscopes come in handy for studying worm activity; worms will move along the surface towards different regions of the dish.

***C. elegans* Studies in Introductory Biology Lab**

Understanding how *C. elegans* interacts with organisms in its natural habitat is a relatively recent addition to *C. elegans* research. Most of our understanding of *C. elegans* comes from the laboratory, where *C. elegans* lives on Petri dishes containing sterile media and a single strain of the bacterium *Escherichia coli* (*E. coli* OP50) as a food source. Over 25,000 articles have been published on *C. elegans* in neurobiology, genetics, developmental, and cell biology. Interestingly, because *C. elegans* is a model for research, it was the first multicellular organism to have its genome sequenced.

In this Unit, you will design experiments to evaluate how *C. elegans* interacts with chemicals and bacteria that are potentially found in its natural environment. Many of the experiments that you design will be testing questions that have never been tested. There is no "right" answer. You may find this challenging, but science can be incredibly exciting and rewarding when you do not know the answer beforehand.

The first two labs of this unit you will get to know the worms, practice working with them, and learn how to design and analyze chemotaxis experiments. In these labs you will study *C. elegans* and their attraction to/repulsion from various volatile chemicals.

You will then have three lab periods where you are able to design your own experiment pertaining to *C. elegans* (it is these 3 labs that you will use for your research paper). For these labs you will have access to wild type and mutant *C. elegans*, volatile chemicals and a range of bacterial species. We are also happy to try and track down any other resources that you request. Be creative in your thinking here and have some fun – we don't know what you will choose to research and we don't know what your results 'should' be but if you choose an interesting question these labs will be fun and could lead to novel scientific findings!

Learning Objectives:

The lab has a number of learning goals. In particular, after completing this lab, you should be able to:

- Use Web of Science and appropriate search terms in order to find publications that will help you define your research question
- Formulate a novel hypothesis and design a feasible experiment to test that hypothesis.
- Recognize why controls are important for science in order to be able to assess whether an experiment has the appropriate controls.
- Critique alternative experimental approaches and refine experimental designs based on results from previous experiments.
- Interpret scientific data using statistical methods in order to determine whether the formulated hypothesis is supported or rejected.
- Recognize and define sources of data variability in an experiment.
- Create graphical depictions of data in order to visualize data variability.

- Evaluate data in an ecological and evolutionary context.
- Write a scientific research report and give an effective oral research presentation.

Available Reagents:

Chemicals:

4-heptanol
1-heptanol
isopropanol
isoamylalcohol
acetone
diacetyl
ethanol
methanolmenthol
benzaldehyde

Bacteria:

Escherichia coli, strain OP50. This is the common strain of *E. coli* used to feed *C. elegans* in the lab. We refer to it as OP50. All your worms have fed on this previously. OP50 is a slow-growing uracil dependent strain.

A common strain of non-pathogenic *E. coli* used in the lab. *C. elegans* fed HB101 have been shown to reach adulthood faster than *C. elegans* fed OP50 *E. coli* (So *et. al* 2011).

Bacillus subtilis. Found both in soil and in the human gut this gram-positive bacterium can live in extreme environmental conditions and has been used to stimulate the immune system.

Bacillus cereus. A soil-dwelling bacterium that can also live in the gut and has been used as a probiotic for chickens to reduce other gut flora.

Bacillus thuringienesis. Although safe for humans this bacteria can be used as an insecticide against caterpillars.

Serratia marsecens. A potential human pathogen this bacterium is red in color due to production of prodigiosin.

Burkholderia cepacia. A gram negative bacterium typically found in water or soil that can cause pneumonia in cystic fibrosis patients.

Enterobacter aerogenes. A gram-negative bacterium that is found in soil, water, and the human gut that can cause infection in immunocompromised individuals.

Rhizobium leguminosarum. An endosymbiotic gram-negative bacterium found in plant cells that can fix nitrogen.

Sporosarcina ureae. Found in soil, especially soil high in urine content as it is capable of metabolizing urea.

Worm Strains:

Strain Wildtype (WT). This worm strain is a good control worm. It has no known mutations in genes that might impact motility or feeding behavior. This is the strain you will use in the first chemotaxis experiment. You will likely use it in all of your experiments on at least some of your plates.

Strain CB3330. Mutation in gene che-11, has defects in cilia that impact movement.
Strain CB3329. Mutation in gene che-10, has defects in cilia that impact movement.
Strain CX2357. Mutation in gene odr-5, an odorant receptor gene.
Strain CX4148. Mutation in gene npr-1.
Strain CX4. Mutation in gene odr-7, an odorant receptor gene.
Strain PR671. Mutation in gene tax-2, leading to multiple sensory defects.
Strain PR675. Mutation in gene tax-6, leading to multiple sensory defects.
Strain PR678. Mutation in gene tax-4, leading to multiple sensory defects.

Potential Research Areas for Worm Choice Experiments

You will work in groups to design and conduct experiments to study the behavioral ecology of *C. elegans*. Because the scientific process starts with creativity and ideas, it is important that you come up with your own ideas. However, projects should generally fall within the scope of feeding and disease avoidance behavior of *C. elegans*, and can be centered around topics listed below. Furthermore, you will need to use available resources and will be expected to refine your design based on input from your classmates and instructors.

1. Food Choice

In their natural environment, C. elegans are exposed to a plethora of bacteria; some are food, but others are pathogenic. How do worms deal with the variety of bacteria? Do they preferentially seek out particular bacteria for food? Food choice experiments can be done to ask several questions:

How do natural diets compare to lab diet? Worms are often fed the standard *E. coli* strain OP50, which was chosen to facilitate lab experiments. But how does this standard lab diet compare to bacteria that they might encounter in the soil and plants where they naturally dwell? These experiments require no-choice tests: rearing worms on plates with different types of bacteria.

Can worms distinguish between different bacterial diets? We can hypothesize that worms may be able to seek out what is best for them. We can test this by carrying out choice tests: worms are provided different diets on different sides of their agar plates.

2. Pathogen Avoidance

Bacteria do not just provide food to worms; they also can pose health hazards. Many bacteria cause disease, and worms that can avoid getting sick should have an advantage in nature.

Compare the performance of worms in the presence of different pathogens. How does exposure to *Serratia marcescens* affect worm survival? How about *Bacillus thuringiensis*?

When given a choice between different bacteria, can worms actively avoid pathogens?

Is pathogen avoidance dependent on particular worm traits? For example, do worms with mutations in genes associated with smelling no longer avoid pathogens?

3. Chemicals in the Environment

Worms are also exposed to many different chemicals in their environments, and man-made pollution has the potential to interfere with worm well-being.

Are worms attracted or repelled by certain chemicals? Do they avoid contact with chemicals that are harmful; do they seek out chemicals that may indicate that there is food?

How do different chemicals affect the survival of worms? We can directly compare the performance of worms using different chemicals available in the lab.

4. Mutant Studies

Worms are cool. One of the reasons they are cool is that many different mutants are available. We can even order them online! Take advantage of this to explore the mechanisms by which worms are attracted to or repelled by certain bacteria and chemicals. What role does smell play? Or taste? Or the ability to move quickly?

C. elegans References to Get You Started

This is a list of references to help get you started.

Worm Book. Your worm bible. This website includes downloadable chapters on worm ecology, evolution, behavior and neurobiology. These are peer-reviewed articles that you can cite as primary literature in your presentations and research papers.

Worm Atlas. Another great worm website.

Chang, H. C., J. Paek, and D. H. Kim. 2011. Natural polymorphisms in *C. elegans* HECW-1 E3 ligase affect pathogen avoidance behaviour. *Nature* 480: 525-529. Paper investigated natural variation in avoidance of *Pseudomonas aeruginosa*, then used genetic mapping to identify genes.

Coolan, J. D., K. L. Jones, T. C. Todd, B. C. Carr, and M. A. Herman. 2009. *Caenorhabditis elegans* genomic response to soil bacterial predicts environment-specific genetic effects on life history traits. *PLoS Genetics* 5(6): e1000503. Identified 21 genes that impact fitness or lifespan in particular bacteria environments.

Laws, T. R., H. S. Atkins, T. P. Atkins, and R. W. Titball. 2006. The pathogen *Pseudomonas aeruginosa* negatively affects the attraction response of the nematode *Caenorhabditis elegans* to bacteria. *Microbial Pathogenesis* 40: 293-297.

Pradel, E., Z. Zhang, N. Pujol, T. Matsuyama, C. I. Bargmann, and J. J. Ewbank. 2007. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcesans* by *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences* 104(7): 2295-2300. Worms exit bacterial lawns of *S. marcesans* a few hours after entering it. Lawn avoidance involves several nematode genes.

Reddy, K. C., E. C. Anderson, L. Kruglyak, and D. H. Kim. 2009. A polymorphism in npr-1 is a behavioral determinant of pathogen susceptibility in *C. elegans*. *Science* 323: 382-384. NPR-1 mediated resistance is through oxygen-dependent behavioral avoidance rather than direct regulation of innate immunity.

Schulenburg, H. and J. J. Ewbank. 2007. The genetics of pathogen avoidance in *Caenorhabditis elegans*. *Molecular Microbiology* 66(3): 563-570.
Shtonda, B. B. and L. Avery. 2006. Dietary choice behavior in *Caenorhabditis elegans*. *The Journal of Experimental Biology* 209: 89-102.
So, S., K. Miyahara, and Y. Ohshima. 2011. Control of body size in *C. elegans* dependent on food and insulin/iGF-1 signal. *Genes to Cells* 16:639-51.
Zhang, Y., H. Lu, and C. I. Bargmann. 2005. Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature* 438: 179-184. Paper shows good example of choice assay design.

Worm Protocols

Labeling Plates:

- Use sharpie markers to label your plates on the bottom (side with agar) and at the edge of the plate so as not to obscure your view of the plate
- You should include things like where worms and chemicals will be plated, regions where you will count worms, chemical plated, plate #, etc.
- For plates that will be kept after lab you should include additional info. Like your lab group name, lab day/time/room, etc.

Collecting and Washing Worms:

- Wear gloves
- Take the lid off your master worm plate and tilt it 45 degrees
- Gently pipette 1mL of M9 buffer over a worm 'spot', collect and repeat 4-5 more times
- Move worms and liquid to an eppendorf tube and collect worms in the bottom by centrifuging 1-2sec. at a time
- Pipette off most of the liquid (supernatent) without disturbing your worm pellet and save this just in case anything goes wrong
- Add 1mL M9 buffer to your worm pellet and flick to mix the worms (do not vortex). Pellet and remove supernatent again.
- Repeat to wash worms once more
- Resuspend 'clean' worm pellet in 100ul M9 buffer by flicking
- Measure your worm concentration by counting the worms in 5ul of your solution (mix and put 5uL onto a microscope slide and count them using a dissecting microscope)
- Adjust so that you have 3-8 worms/uL (15-40 in 5uL)
- Plate worms by pipetting 5uL onto a worm plate (be sure to mix the solution immediately before pipetting as the worms will quickly settle to the bottom of the tube). Cut the tip off the p20 disposable tip so as not to shear the worms as they pass through the small opening.

Plating Chemicals on Your Plates:

- Chemicals will evaporate quickly so should not be added to a plate until after the worms are added and the droplet of worms has dried. You should add chemicals in the fume hood. Plate 2ul of a chemical or 2ul of a dilution of a chemical.

Plating Bacteria on Your Plates:

- Plate 5uL of a bacteria liquid stock onto plates (remember to vortex before removing the bacteria as they will settle to the bottom of the tube) and allow to grow at room temperature for 1 week or at 37 for 2 days before plating worms.

Collecting Worm Data:

- You should make sure all lab partners count worms as uniformly as possible
- You should count worms right after plating so you know how many were added to the plate
- You should note any additional information that you think may be relevant (odd behavior of worms, location close to edge of region, actively moving or not, etc.
- You should record worm data at multiple (at least 3) time points
- You should include replicate data sets (a minimum of 6 replicates for any plate design is a good starting point)

Data Analysis:

- Treat each plate as a replicate (this will help control for any 'bad' plates due to contamination, poor bacterial growth, etc.).
- It is best to use proportions of worms instead of straight counts to control for different numbers of worms on different plates.
- One sample T-tests can be used to analyze data gathered from a single plate design. For example, a one sample T-test could be used to determine if *C. elegans* have a significant preference for OP50 bacteria over *B. subtilis* bacteria when given the choice on a single plate.
- Two sample T-tests can be used to analyze data gathered from different plate designs. For example, a two sample T-test could be used to determine if wild type and mutant *C. elegans* have different preferences for OP50 versus *B. subtilis* bacteria when given the choice on a single plate. Here, there are two plate designs as one set of plates uses wild type worms while the other uses a mutant strain.

Materials

Student Materials

- Stocks of various chemicals (student groups will only use small ~25uL quantities of any chemical they choose to use). Hazardous chemicals should be kept in a fume hood.
- Stocks of various bacteria as liquid cultures aliquoted in the fridge (student groups will only use small ~50uL quantities of any bacteria they choose to use).
- Master plates of *C. elegans* worms for students grown in advance of the lab. One master plate for each lab group for each strain they want to use (can restrict this to only wild type *C. elegans*). Master plates can be prepared by cutting up a current master plate of worms and placing 1 'chunk' onto a fresh plate with pregrown spots of OP50 bacteria. For more in-depth projects where students may want to use mutant worm strains it can be helpful to require them to submit a request for each strain they will need in advance. Worm strains can be frozen and reanimated and expanded before the lab leaving enough expansion time to obtain the necessary numbers of master plates.
- Worm NGM plates – we find at least 12 per lab group per lab period is necessary to give students enough to include needed replicates and controls.
- Student dissecting microscopes to view and count worms
- 15mL of M9 buffer (used to wash and plate worms) per lab group.
- OP50 *E. coli* bacteria (used as a food source to maintain *C. elegans* stocks)
- Micropipettes & tips
- Gloves & biohazard waste
- Sharpie markers to label plates

Obtaining *C. elegans*

Mail requests for worm strains to *Caenorhabditis* Genetics Center, University of Minnesota, 250 Biological Sciences Center, 1445 Gortner Avenue, St. Paul, MN 55108-1095 USA.

Nematode Growth Medium (NGM) Plates

1. Mix 3 g NaCl, 17 g agar, and 2.5 g peptone in a 2 litre Erlenmeyer flask. Add 975 ml H_2O. Cover mouth of flask with aluminum foil. Autoclave for 50 min.
2. Cool flask in 55°C water bath for 15 min.
3. Add 1 ml 1 M $CaCl_2$, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$ and 25 ml 1 M KPO_4 buffer. Swirl to mix well.
4. Using sterile procedures, dispense the NGM solution into petri plates using a peristaltic pump. Fill plates 2/3 full of agar.
5. Leave plates at room temperature for 2-3 days before use to allow for detection of contaminants, and to allow excess moisture to evaporate. Plates stored in an air-tight container at room temperature will be usable for several weeks.

Seeding NGM plates for a Master Plate

1. Drop 50ul of an overnight culture of *E. coli* OP50 bacteria onto an NGM plate. OP50 can be grown in LB media.
2. Allow to grow overnight at 37°C
3. Add *C. elegans* to the plate by 'chunking'
   a. Cut a previous NGM plate with lots of *C elegans* worms into small .5inch pieces and put one piece on a new NGM plate seeded with OP50. The worms from the chunk will crawl off the chunk onto the new NGM plate.

Maintaining *C. elegans*

1. *C. elegans* are usually maintained on NGM plates with OP50 bacteria at 20 degrees.
2. They will need to be chunked onto fresh NGM-OP50 plates every few days (transfer when they run out of bacteria to eat or have hundreds and hundreds of worms on a plate.
3. Stock plates of *C. elegans* can be kept at 12 degrees for a few months if the plate is wrapped in parafilm to avoid drying out.

Worm M9 Buffer

3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre.

Sterilize by autoclaving.

Additional Information on Maintaining *C. elegans*

http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html

Notes for the Instructor

Overview

This entire lab module was designed to give students the opportunity to design their own hypothesis-driven experiments using live organisms that exhibit interesting behaviors. These behaviors are shaped by the ecological conditions of the worms and by the evolutionary pressures that all animals face in order to feed and avoid disease.

C. elegans are attracted to and repelled by many chemicals and bacteria under laboratory conditions. Much

of these behaviors are shaped by the need for worms to find food (bacteria) and to avoid dying by eating pathogens. We know the worms are also attracted to some pathogens, with deadly consequences. This is costly to the worms but of benefit to the pathogens if the worms spread the pathogens and/or provide the pathogens with additional host resources.

Logistics

This lab can be adapted to run in a single 2- or 3-hour lab period or to span multiple lab periods with the main difference between timeframes being the level of student-led inquiry allowed. We currently run the module over 5 lab periods where the first two periods are spent studying chemical chemotaxis and learning how to handle the worms. The following 3 lab periods are used by students to run an experiment addressing a question of their own design. The repetitive weeks allow room for failure due to technical issues or poor experimental design.

Our students work in groups of 4 for this module to allow for scientific discourse on experimental design and data analysis. Our labs are often open for 2 days following a lab period to allow students to come in on their own time and collect additional time point data. Assessments include short 5-minute presentations, a research paper, worksheets (on experimental plans and data analysis) and quizzes.

Guiding Rather than Directing Experiments

This experience is about guiding the experimental design process rather than lecturing or telling the students the right answer. If they come up with something that is different but should work ok, let them try it. If they are really excited about trying something that is likely to fail, let them try that too.

We have given students only brief descriptions of how to design an experiment and hope that they will come up with many designs. As a class, have students share their designs. Talk about the controls, replication and the challenge of trying to pick the appropriate measurements.

Things You Can Do To Strengthen Your Leadership in This Project

1. Read some of the references listed in the lab module. *C. elegans* have amazing interactions with bacteria. The more you read, the more you may come up with ideas that can help students shape interesting experiments.
2. Guide students towards simple experimental designs.
3. If students are committed to trying something hard, let them do it. So, it might not work. The best science often comes from trying things that are likely not to work.
4. Explain the importance of experimental replication in science. There is a reason that we do experiments over and over again.
5. Get students excited. This is cool.

Common Technical Issues Encountered

Master Plates

It is important to supply the students with master plates teaming with worms so that they can obtain enough worms after washing. It is also easier for the students to wash the worms if the worms are larger and more uniform in size. Timing of when new master plates are chunked can help ensure the students will have optimal plates.

Washing Worms

Many students will struggle in the first lab period (or two periods) simply to properly wash and plate their worms. Common mistakes made by students include improper pipetting, vortexing the worms, not cutting off the tip of a p20 before using, removing too much supernatant and thus worms, and not mixing the worm solution well enough before removing an aliquot. Most groups are able to correct their mistakes after 2-3 tries but some need close watching in order to identify where they are going wrong. Students that struggle beyond a few tries should be guided towards a trouble shooting mode where they count their worm concentration in their solution after each step to identify where in the protocol they lose their worms.

Finding Worms

Many students struggle to find their worms in the microscope especially when using one of their experimental plates with an unknown number of worms. One tip you can share with them is to first focus the microscope using a master plate of worms that is teaming with worms – that way they are sure to have worms in their field of vision that they can focus on – and to then switch out the plate with their experimental plate without adjusting the microscope settings. From this they will generally be in the right focal plane to find a worm and can then adjust settings to focus more clearly on the worms.

Measuring Worm Behavior

Students often have difficulty determining a successful strategy to quantify worm behavior. Issues with original plans are often identified at the end of the first lab period or beginning of the second when students have to discuss their data. A class discussion can be used to guide students to proper data collection techniques. While there are numerous potential ways to quantify worm behavior most students will settle on a simple choice plate design where worms are plated in the center of the plate, chemicals/bacteria are plated a set distance

away and worms are tallied based on entering a zone of proximity around the chemical/bacteria spot.

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