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Testing Hypotheses of Aging in the Nematode *Caenorhabditis elegans*

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A common characteristic of many, if not all metazoans, is aging. Countless evolutionary, molecular, and genetic mechanisms have been described that account for aging of creatures as diverse as yeast and man. This laboratory will put some of these factors to the test; participants will develop a hypothesis that explains the interactions between genes and the environment, and design and implement an experiment to test the hypothesis. Students design controlled experiments using the model organisms *C. elegans* that include variables of environment, food, or genetic background. After completing the experiment, students analyze their data and write a research article-style report.

Keywords: *Caenorhabditis elegans*, aging, lifespan, longevity, genetics, hypothesis, nematode, statistics

Introduction

Objectives

- Students will understand environmental and genetic factors of aging.
- Students will synthesize an original hypothesis concerning an experiment testing longevity that they design.
- Students will collect data, analyze the data, and provide realistic conclusions from their experiment.

Background Information

The factors involved in aging and longevity of organisms are a fascinating topic involving evolutionary theory, genetics, and environmental influence investigation. Almost all animals exhibit the hallmarks of aging: decreased reproductive ability, slower reaction time, accumulation of aging pigments called lipofuscin, decreased regenerative ability, and increased susceptibility to disease. *Caenorhabditis elegans*, *Drosophila melanogaster* (common fruit fly), and *Mus musculus* (house mouse) have been excellent model organisms for the discovery of aging-related genes. In *C. elegans*, the rate of aging is modified by two energy-sensing pathways, insulin signaling and TOR (Target of Rapamycin) (Vellai et al., 2003). The first genes shown to modulate aging in *C. elegans* belong to the Dauer commitment pathway, *age-1*, *daf-2*, and *daf-16*, many of which are involved in the insulin signaling pathway (Kimura et al., 1997; Lin et al., 1997; Tissenbaum and Ruvkun, 1998). Genes that are activated by the insulin-signaling pathway include free-radical scavenger genes and metabolic genes such as sod-3 and fat-7.

Caenorhabditis elegans, a small free-living nematode, is ideally suited to longevity studies for the following reasons. It is easy to culture and manipulate, has a relatively short and consistent life span of 2-3 weeks, has many genetic and environmental influences known that dramatically extend or shorten its life span, and has been adopted as a major model research organism, which ensures that there are plentiful resources available to facilitate its use. Logistically, *C. elegans* is small and easy to maintain; adults are 1 mm long, it is a harmless, free living nematode, and it grows well at temperatures of 20-25˚C. Cultures of worms are easily maintained on small agar plates inoculated with a non-pathogenic, laboratory strain of *E. coli*. *C. elegans* can be viewed easily using a 40X stereo-zoom microscope, and the learning curve to effectively transfer individual worms between plates using platinum wire worm picks has been rapid with students.

Longevity studies are well suited for student laboratories because they are simple to design, inexpensive to implement, and can teach genetics, toxicology, and statistics. Ideally, students design their own experiments within the constraints of the materials provided. Students are responsible for experimental set-up, observation of the worms, analysis of data, and the presentation of results once the experiment is completed. This is a multi-week laboratory that works best if students can access the lab multiple times per week to record results.

One of the strengths of this laboratory exercise is that students synthesize a hypothesis and test it using different genetic *C. elegans* strains and environmental conditions. An additional parameter that can be included is RNA interfer-

ence (RNAi) (Fire et al., 1998; Hannon, 2002), which expands the possible phenotypes students can explore, as well as teaching students about different mechanisms of gene regulation. I have opted to omit RNAi from this laboratory protocol; however, I have used RNAi by feeding special bacteria in my labs and had satisfactory results. The data collected are used to teach statistics, literature review, and critical thinking. My students are first year undergraduates who have been in an aging course for four weeks before designing their experiments; however, these experiments could be incorporated into any course if an hour were given to provide background. The initial laboratory procedure is fairly quick in this implementation, leaving adequate time for discussion.

Previously, my students investigated aging with the addition of resveratrol, which has been shown to extend lifespan in mice (Baur et al., 2006), vitamin E (Harrington and Harley, 1988), vitamin C, a low calorie diet (Zheng et al., 2005), as well as with reduction of the superoxide dismutase (Honda and Honda, 1999) or fatty acid desaturase (Brock et al., 2007). Students were very excited by the project, and although it required them to make observations outside of class for two weeks, most reliably collected data.

Time and Equipment Requirements

This laboratory follows individual *C. elegans* nematodes until their death; therefore, it requires a minimum of three weeks to complete, with student involvement at least three times per week with a duration of approximately 15 minutes. In addition, this laboratory is much easier for the students if under-lit, minimum 40X magnification zoom stereomicroscopes are used. An important aspect of this lab is that is can be set up as a simple experiment for larger courses, or be implemented as an involved project for smaller, more advanced classes.

Student Outline

Almost all animals exhibit the hallmarks of aging: decreased reproductive ability, slower reaction time, accumulation of damaged proteins, decreased regenerative ability, and increased susceptibility to disease. Interestingly, the rate that this occurs appears to have both genetic and environmental influences. Nematodes, fruit flies, and mice have been excellent organisms for the discovery of aging-related genes. In the non-parasitic, microscopic nematode *C. elegans,* the rate of aging is normally 2-3 weeks, but can be extended for months (Riddle 1977).

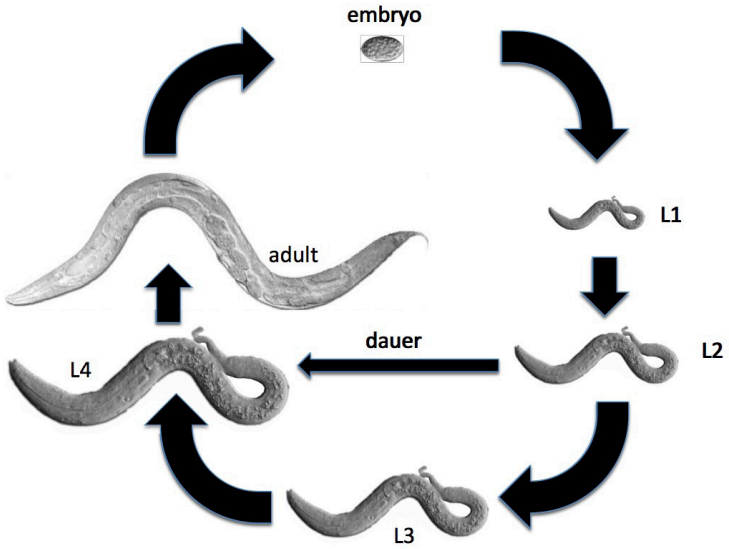

Figure 1. *C. elegans* life-cycle

C. elegans are classified as Ecdysozoa, a clade of animals that molt; well-fed *C. elegans* has four distinct molts (Fig. 1), L1-L2, L2-L3, L3-L4, and L4-adult (Aguinaldo et al., 1997). These worms generally live about 2 weeks, most of it as adults, and then spontaneously die (Fig. 2). However, worms under severe caloric restriction or overcrowding may make an alternative L2-L3 molt to form a Dauer larva (Riddle, 1988). Dauer larvae are distinct from other larval stages in that the digestive system is sealed off from the environment by skin, and that these worms can survive many months without food, extending the worms' lifespan up to 6-fold. Interestingly, activation of some of the Dauer genes in adult worms also increased their lifespan.

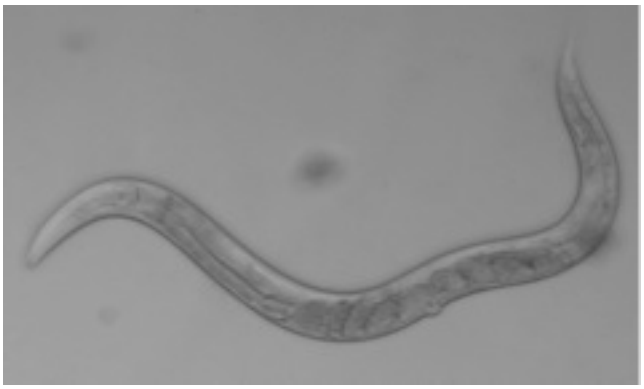

Figure 2. *C. elegans* adult hermaphrodite

In *C. elegans*, the insulin-signaling pathway is responsible for both longevity and for entry into the environmentally resistant Dauer (Fig. 3). Many mutants in the Dauer control genetic pathway are said to be Daf, or Dauer-defective. The best studied of these genes, *daf-2*, *daf-16*, and *age-1* have been shown to be homologous to the insulin/IGF-signaling pathway of mammals (Kimura et al., 1997; Lin et al.,1997; Tissenbaum and Ruvkun, 1998): *daf-2* is homologous to the mammalian insulin receptor, and its activation reduces lifespan; *daf-16* encodes a transcription factor that has been shown to target many cellular repair and protection genes; *daf-16* is negatively regulated by the insulin-signaling pathway. Activation of the *daf-16* genes increases longevity and is essential for dauer morphogenesis. *age-1* encodes a protein kinase that transduces the *daf-2* insulin receptor signal to *daf-16*.

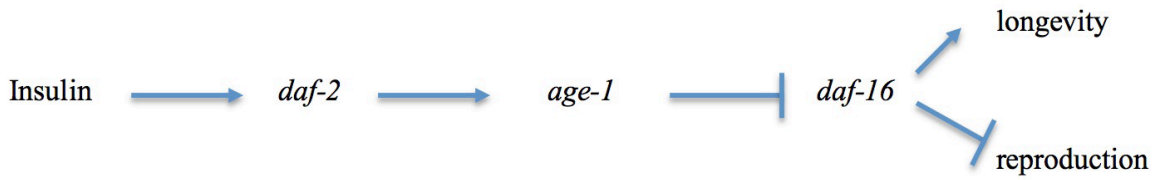

Figure 3. The insulin-signaling pathway in *C. elegans* affects life span and reproduction

Beyond insulin signaling, the sirtuin genes, temperature fluctuations, resveratrol, antioxidants, designer superoxide dismutase mimetics, and the success of reproduction have all been correlated with changes in longevity and aging.

This is a multi-week laboratory that will require accurate data recording.

Week 1: Prelab

Objective

Create a hypothesis of the effect of an environmental condition of the roundworm *C. elegans* on the mean longevity of a population.

Background

You will be performing an aging lab using *Caenorhabditis elegans* as a model for animal longevity. *C. elegans* is a very small, free-living nematode. You will observe *C. elegans* using stereomicroscopes and manipulate them using a small platinum wire. *C. elegans* eats bacteria such as *E. coli*, which are grown on small Petri dishes containing a medium called Nematode Growth Medium (NGM). Growing in different environmental conditions is simple, making them ideal for many types of experiments. Normally, *C. elegans* lives 2 - 3 weeks; however, mutations and environmental conditions can affect *C. elegans* life span

Hypothesis

You should prepare a proposal describing the experimental conditions, hypothesis, and rationale for your hypothesis. The hypothesis should be based on prior readings, future readings, or other sources. Of course, the best experiment to propose is the one that interests you!

The design of all experiments will include:

1. Selecting a worm strain or strains
2. Selecting a treatment,
3. Selecting appropriate controls*

*Remember, a controlled experiment has a control for each variable tested.

Worms of a specific life stage (L2 - L4) will be placed on Petri dishes prepared with the experimental chemical or condition. Generally, worms are stage synchronized by a bleaching protocol that eliminates all organisms other than *C. elegans* embryos. You will be provided hatched embryos that are all approximately the same age. A chemical called FUDR will also be incorporated into the food of the worms; FUDR prevents the worms from reproducing without causing sterility (*why is reproduction disadvantageous in this experiment?*). Once the worms are no longer reproducing, you will observe the plates daily and count how many worms are surviving. This will be your primary source of data.

Select from the Following Experimental Conditions:

Worm strains

A worm strain has a defined genotype and phenotype. This is a genetic determinant of aging; the DNA of a worm strain contains specific, non-wildtype alleles.

- ***daf-2*** Insulin signaling/dauer – *daf-2* worms cannot receive insulin signals
- ***daf-16*** Insulin signaling/dauer – *daf-16* worms cannot become Dauers
- ***age-1*** Insulin signaling/dauer – *age-1* worms cannot transduce an insulin signal to the nucleus
- N2 Wild-type worm strain – normal insulin signaling

Treatment

You can alter the amount of food on a plate (or remove all food), or add/remove specific nutrients. Generally, *C. elegans* are grown on NGM plates which contain nutrients allowing for additional bacterial growth. No Calorie plates may be used to prevent *E. coli* growth for no or low food experiments. In addition, you can add antioxidants or small molecules that affect aging in yeast or mice. You could add pollutants or pro-aging compounds as well.

- **Food** lack of or extra quantities of food
- **Cholesterol** *C. elegans* requires cholesterol in its food
- **Glucose** energy molecule (worms eat bacteria, so it may not be relevant)
- **Chemicals** examples: caffeine, alcohol, smoke captured in liquid, or others
- **Resveratrol** small molecule that interacts with aging found in red wine
- **Vitamin C** antioxidant
- **Vitamin E** antioxidant
- **Red wine** a component of the "French Paradox"
- **Light** Light cycle variations (but remember, worms don't have eyes)

Think about the how these conditions or chemicals might increase or decrease the normal 2 - 3 week lifespan of *C. elegans*. Also, provide a rationale for your hypothesis on the sheet provided in this laboratory exercise. Submit your experiment sheet to your instructor. See Appendix D and Table 1.

Table 1. Sample of your experimental proposal.

Hypothesis: N2 Strain worms living on vitamin C + vitamin E plates will live longer than worms on either vitamin C or normal control plates.		
Rationale: Vitamin C and E are antioxidants. Having more antioxidants will protect the worms from damage from oxygen.		
Experiment	**Strain**	**Condition**
Control	_____N2 wildtype _____	___ normal NGM___
Experimental Group 1	_____N2 wildtype _____	___ Vitamin C _____
Experimental Group 2	_____N2 wildtype _____	___ Vitamin E _____
Experimental Group 3	_____N2 wildtype _____	___ Vitamin C + E __

Week 2

Objective

Set up experiment by placing the nematodes on the correct plates.

Protocol

Become familiar with worms by moving a few worms to fresh plates

1. Moving microscopic worms with a microscope requires some practice; however, most people get the hang of it relatively quickly.
2. Sterilize the platinum wire worm pick using the flame of an alcohol lamp. The tip of the wire should briefly glow orange/red. Remove the pick from the flame; it generally cools completely in a matter of seconds.
3. Using a NGM agar plate with OP50 *E. coli* reserved for this purpose, gently scoop some of the bacteria onto the end of the worm pick. These bacteria will act as an adhesive for the next step; therefore, it is often referred to as "sticky" bacteria.
4. Using a stereomicroscope, gently place the pick with sticky bacteria upon the desired worm. The worms will generally stick to the bacteria and will be attached to the pick.
5. Using the stereomicroscope, gently place the tip of the pick on the agar surface of the destination plate. Do not try to force the worm off, rather, hold e pick still, enabling the worm to crawl off by creating a continuous surface between the sticky food on the pick and the agar surface.

Begin your Project

1. Your instructor will come by your group and discuss your project.
2. Obtain NGM or No-Calorie plates for your experiment. You will need two plates for each condition and control you are testing as well as an extra test plate to count worm culture density.
3. Add 0.2 ml of any test solution required for your experiment, i.e. add 0.2 ml of sterile Red Wine to plates testing Red Wine or 0.2 ml of Vitamin C solution to plates testing Vitamin C.
4. Be sure to label the SIDE or BOTTOM EDGE of the plate with your name, the date, a unique number, and the experimental condition or control you are testing. It is best to write along the edges in small writing.
5. There will be stage-synchronous cultures of *C. elegans* in ice buckets at the front of the room of the N2, *daf-2*, *daf-16*, and *age-1* strains on NGM FUDR plates.
6. Add 1.0 mL of sterile H_2O to a plate of provided synchronized worms and gently swirl and tilt to dislodge the worms from the surface.
7. Transfer ~ 0.1 mL of the dislodged worm solution to a test plate; the goal is to have 25 - 50 worms transferred, but the number might be different. Count the worms on the test plate. Calculate the amount of dislodged worm liquid that will need to be transferred to your experimental and control plate to obtain 25 - 50 worms on each.
8. Using a stereo zoom microscope, count the number of worms you have transferred and record the number as your starting number (n).
9. Place the plates in your plastic box and move them to a 24˚C incubator.

Weeks 1-4

Objective

Collect longevity data for your worms by frequent observation.

Protocol

- See **Collection of Data** section.
- Worm plates should be examined and the surviving *C. elegans* counted every 2 to 3 days.
- Retrieve your worm plates and observe the worms under the dissecting microscope.
- For each plate, count the total number of moving worms as living and record this, and any other observations in your notebook.
- An eyelash pick can be used to gently prod still worms to determine if they still can move.
- Replace the worms into the plastic box and return the box to the incubator.
- Continue repeating this procedure every 2 to 3 days, until the project ends or all of your worms are dead.

Collection of Data

You and your lab partner should devise a schedule allowing observation and counting of the worms every other day. Record these data for each group until all worms have died or the time allotted for this laboratory ends (Table 2). You may also record qualitative traits such as the relative size, color, or movement characteristics of the worms.

Table 2. Example of Data Collection for Aging Laboratory

Genotype	Condition	Date									
		3/13	3/15	3/17	3/20	3/22	3/24	3/27	3/29	3/31	4/3
wildtype	normal	30	30	30	29	27	25	19	10	2	0
wildtype	Vitamin C	30	28	27	26	26	24	21	13	3	1
wildtype	Vitamin E	30	29	29	29	28	27	20	15	6	1
wildtype	Vitamin C + Vitamin E	30	30	30	30	28	26	21	15	12	10

Writing the Lab Report

The report should be written as a research article. You will need to cite at least three credible sources for information in the report. These sources may be review papers, primary articles, or online public databases. You should also use lecture materials that are relevant to your research topic or materials; be sure to use proper citation style. The expected size of the paper is approximately four to five double-spaced typed pages; but length is less important than content (I will evaluate on completeness, not on length).

Each pair will provide one lab report, unless the students of the group would prefer to each write their own. To account for differences in effort by group members, an individual evaluation sheet will be provided for each student to rate his/her own performance as well as his/her lab partner's involvement throughout the project. This evaluation will be taken into account when assigning grades for written report.

Provide a Title page with the following information:

1. **Your Names**
2. **Title** – a descriptive title of the project; it should include the organism name and the project's purpose or procedure.
3. **Date**

The report should include the following sections:

Introduction

Include information that explains why the gene or conditions you have chosen for your experiments are appropriate, i.e., how do these conditions relate to aging of organisms. Refer to outside sources when stating facts or conclusions of other researchers' work. You are permitted to cite papers or books read in this class, as well as lecture material if you have accurate notes. You will want to define any specialized scientific terminology (jargon) you will use in your report. Many papers will be enhanced with a figure describing the problem to be addressed. The introduction should lead up to your hypothesis, allowing the reader to understand this logical prediction for the tests you are doing. (estimate: 1 ½ to 2 pages)

Hypothesis

Explicitly state your hypothesis or hypotheses. Provide an additional sentence or two to justify this hypothesis if it is not absolutely clear from the introduction.

Materials and Methods

Describe the worm strains and types of agar plates you used for your experiment(s). Briefly describe your procedure, such as how often you moved worms, how you counted worms, the target number of worms to study, and special conditions, but do not go into detail about the number of worms moved on each day, etc… Enhancements to materials and methods would include a flow chart or diagram of the experimental procedure. The goal of the material and methods section is to allow a competent scientist to repeat your experiment. (estimate: 2 to 3 paragraphs)

Observations/Analysis

Provide your data in table and graph format. Collection of data is one step in addressing your hypothesis. Analyze the data to observe differences and trends. Graphical representation of data generally helps a researcher, or reader of the research, compare absolute numbers and trends more easily than tables. In aging research, longevity is generally plotted on a line graph with time being the independent axis and survival the dependent axis. These survival curves, such as a Kaplan-Meier plot, allow two or more experimental conditions to be compared, with both slope and percentage distance between the two points obvious to the observer. Other types of data may be graphed using histograms or pie charts, such as activity levels between groups, size, or the number of individuals surviving at a point in time. In addition, statistical analysis is used to determine if the differences between the Hazard Rate are significant. Generally the log-rank test is employed, which provides a p value. The log rank test can be computed at **http://bioinf.wehi.edu.au/software/russell/logrank/.** See Appendix C for detailed information.

Include sources of error. (estimate: one or more tables showing worm longevity, one or more graphs, probably 1 to 2 pages)

Conclusion/Discussion

State if your hypothesis was supported or refuted. It is also possible that the experiment did not conclusively support or refute your hypothesis (statistics will provide you with that answer). The discussion does allow you a place to provide explanations for trends you observed, even if the data were not significant. From these trends, relate your experiments to the work of other scientists. The conclusion also includes explanations of sources of error and future directions (where do you go from here; even if it is a redesigned experiment). (estimate: 1 to 1 ½ pages)

References

Provide a list of references you used for background, materials and methods, analysis, or discussion. You do need to cite information in your text; if it is not common knowledge that our Art, Communications, English, or Physics majors would not know, it requires citation.

Materials

For 24 Students:

- 12 Zoom stereo microscopes with at least 30X magnification
- 15˚C incubator (useful)
- 25˚C incubator (useful)
- 12 plastic boxes for plate storage
- Parafilm
- 2 feet Platinum/iridium wire
- 1 Box Standard Pasteur pipettes
- 12 Alcohol lamps
- 1 Bunsen burner
- 6 boxes of Tooth picks
- 6 containers of Nail polish
- Sterile transfer pipettes (1 mL with 0.1 mL gradations)
- Worm strains
- *E. coli* (the OP50 strain works best)
- 50 NGM agar plates (60 mm) inoculated with 100 µL of OP50 bacterial solution
- 100 NGM agar plates (60 mm) inoculated with 100 µL of OP50 bacterial solution with FUDR
- 50 Low Calorie Plates with FUDR
- Chemical solutions to be applied to plates

Notes for the Instructor

This laboratory can be performed in multiple, consecutive 50-minute class sessions rather than in a traditional laboratory time block.

Worm cultures

C. elegans reproduce and grow rapidly; always have plenty of worm plates prepared and ready for use. We use a Tritech Research PourBoy 4 automatic plate-pouring machine to ensure our plates have consistent volumes of media and to decrease the incidence of contamination. Plates should be allowed to sit 24 hours before being seeded with OP50 bacteria, and then should be left at room temperature for ~ 1 week to allow them to dry a little for better absorption of chemicals the students choose to add to the experiment.

Bacterial and fungal contamination are detrimental to worm experiments. Always use sterile techniques to prevent the introduction of non-OP50 bacteria or fungal species to the worm cultures. Worm cultures may be cleaned by a bleaching protocol; however, this takes time. The steps in preparing a synchronized *C. elegans* culture outlined in Appendix B will remove contamination from worms before they are distributed to students.

Equipment

Good-quality stereomicroscopes with a bright light source and under stage lighting is extremely helpful in observing the worms. We use Leica S6E, MZ6, and GZ6 series microscopes with an under stage rotating mirror and fiber-optic light source to direct the light for the best contrast of the worms. The procedure will work with less-capable microscopes; however, it will be more difficult for students to observe the worms.

Picking Worms

Students generally learn to pick worms with a platinum wire pick within an hour. In this protocol, students will only need to pick worms and transfer them to a new plate as an exercise; it is not essential to the working of the experiment. It is important to understand worms will stick to the semi-desiccated bacterial glob on the end of the pick, rather than the platinum wire itself.

Safety

1. The *C. elegans* strains are non-pathogenic. They will not survive on skin or lab bench.
2. The OP50 *E. coli* strain is a non-pathogenic lab strain auxotrophic for uracil.
3. The only chemical of concern is FUDR (Floxuridine), used to control reproduction of *C. elegans*. Floxuridine inhibits DNA replication, and is a common chemotherapy drug. Because each worm can normally give birth to up to 300 progeny in a matter of days, without FUDR, the reproductive capacity of a single worm would produce a population crash in two generations. The amount of the compound used in a *C. elegans* plate is far below the level considered therapeutic or dangerous to humans.

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Appendix A

Lab Set-Up Protocols

Creating Eyelash Picks

1. Place a small drop of nail polish on the end of a toothpick
2. Pluck an eyelash or eyebrow and insert the follicular (large) end into the nail polish.
3. Let dry and do not sterilize in a flame.

Creating a Worm Pick

1. Cut a 1.0 cm piece of platinum/iridium wire (Ladd Research, part #30575).
2. Create a shortened glass Pasteur pipette by carefully breaking off most of the thinner glass tip, leaving about 1.0 cm of tip. This should be done carefully by breaking the glass in a paper towel wearing gloves and safety glasses.
3. Insert 1/3 of the wire into a shortened Pasteur pipette, holding the wire in place with a pair of forceps.
4. Place the end of the pipette containing the wire into a gas flame and twist the wire with the forceps to melt the glass and close the end.
5. Let the pick cool and flatten and bend the end of the wire to form a scoop.

Obtaining Worms for the Experiment

C. elegans stocks may be obtained from the *C. elegans* Genetics Center (CGC) at the University of Minnesota (see Appendix B). For this lab, the following strains are useful:

Strain: N2	Genotype: *C. elegans* wild isolate.
Strain: CB1370	Genotype: *daf-2(e1370)III.*
Strain: CF1038	Genotype: *daf-16(mu86) I.*
Strain: TJ1052	Genotype: *age-1(hx546)II.*

Growing Worms

6 weeks pre-lab

1. Order worms strains from CGC if necessary
2. When worms arrive, re-plate some on OP50 seeded NGM plates to start new cultures
3. Transfer 3-5 worms to a new OP50 seeded NGM plate every week to maintain healthy cultures

3 weeks pre-lab

1. Thaw frozen worm strains if already stored on premises
2. Move 3-5 thawed worms to a new OP50 seeded NGM plate 24 hours post-thaw

2 weeks pre-lab

1. Start stock worm plates by adding a small number of worms to an OP50 seeded NGM plate, either by using a worm pick or by diluting worms
2. Store at 20-25˚C
3. Re-plate a small number of worms to new OP50 plates every 5 days to maintain healthy cultures.

5 days pre-lab

1. Cut out (chunk) a 1 cm3 cube of agar from a stock worm plate having many moving worms (worms will be on the agar chunk)

2. Place onto an OP50 bacteria seeded 10 cm NGM plate. Incubate between 20-25˚C for 2-3 days until the plate is covered with eggs and gravid adults.

48 hours pre-lab

1. When the plates are ready, pipette 5mL of M9 solution onto the plate and gently swirl it to suspend the worms.
2. Tilt the plate slightly and transfer the worms to a 15 mL conical tube using a pipette.
3. Bring the volume up to 10 mL with M9 solution.
4. To separate the worms from the liquid, either sediment by gravity by placing the tube in an ice bucket for 15 minutes or centrifuge for about 1 minute using a clinical centrifuge at low-medium speed.
5. Carefully remove as much of the M9 solution as possible without disturbing the worm pellet.
6. Add ~15 mL of 20% alkaline bleach solution to the tube, or as much as will fit.
7. Mix the worms with the bleach solution by inverting gently for 5 minutes. The bleach solution dissolves the larval and adult worms, but the eggshell briefly protects the embryos from destruction. Do not bleach for longer than five minutes or the embryos will be killed by the bleach.
8. Immediately centrifuge at medium speed for 1 minute.
9. Remove as much of the bleach solution as possible by aspirating without disturbing the worm pellet.
10. Rinse the embryos by adding 15 mL of M9 solution to the tube and mixing well.
11. Centrifuge again at medium speed for 1 min.
12. Aspirate the M9 solution without disturbing the embryo pellet, which will be small.
13. Repeat the M9 rinse, centrifuge, and aspiration step at least one more time.
14. Add about 7 mL of fresh M9 and agitate gently to resuspend the pellet.
15. Let the eggs hatch overnight with gentle rocking. Embryos should hatch and remain L1 larvae because no food is present in the M9 solution. This provides a synchronous culture. Larvae will survive up to 48 hours without food.

24 hours pre-lab

1. Transfer worm solution to OP50 seeded FUDR NGM plates ~24 hours before use by students.

Solutions

Making normal NGM agar plates

1. Autoclave the following:

   2.5 g Bacto Peptone
   3.0 g NaCl
   20 g Bacto Agarose
   975 ml distilled H2O

2. After autoclaving, add:

   25 ml 1M Potassium Phosphate buffer, pH 6.0
   1 ml 1M CaCl2
   1 ml 1M MgSO4
   1 ml 10 mg/ml Cholesterol in Ethanol

3. Pour ~10 ml of media into each 60x15mm plastic Petri dish; an automatic plate pouring machine is very useful for making consistent plates
4. Inoculate NGM plates with 50 to 100 µl of fresh OP50 culture and allow to grow overnight.

NGM agar plates + FUDR (to control reproduction)

Follow the recipe for NGM agar plates, with the addition of 100 µL of 100 mM FUDR at Step 2

Low Calorie plates + FUDR

Follow the recipe for NGM agar plates, with the modifications of:
Eliminating the Bacto Peptone (calorie source)
Addition of 100 µL of 100 mM FUDR at step 7

M9 solution (1 Liter)

1. Mix the following:

   5.8 g Na_2HPO_4 $7H_2O$
   3.0 g KH_2PO_4
   5.0 g NaCl
   0.25 g $MgSO_4$ $7H_2O$
   ddH_2O to 1 L

2. Sterile filter (0.22 µm) and bottle

20% Alkaline Hypochlorite Solution (50 mL)

1. Mix the following in a 15 conical tube:

   27.5 mL ddH_2O
   12.5 mL 1 M NaOH
   10.0 mL Bleach (ordinary laundry bleach, regular strength)

This solution should be made fresh for each worm synchronization and preparation protocol.

Concentrations of solutions that are generally useful for experiments.

All solutions should be sterile filtered if possible.
200 µL Red Wine
200 µL of 10 mM vitamin C
The contents of 1 100 IUE capsule of water-soluble vitamin E
200 µL of 10 mM resveratrol
200 µL of 10 mM EUK343
200 µL of 10 mM caffeine in H_2O
200 µL of 50% glucose
200 µL of 50% ethanol

Appendix B

Suppliers

Ladd Research

http://www.laddresearch.com
83 Holly Court
Williston, VT 05495
Phone (800) 451-3406 (USA)
Fax: (802) 660-8859
Part # 30575 - Platinum Iridium Wire 80:20, 8 mil, 2 feet

Sigma

http://www.sigmaaldrich.com/
Sigma-Aldrich
3050 Spruce St.
St. Louis, MO 63103
Item: F0503 - 5-Fluoro-2′-deoxyuridine

***C. elegans* Genetic Center (CGC)**

http://www.cbs.umn.edu/CGC/
University of Minnesota, Dept. of GCD
6-160 Jackson Hall
321 Church Street S.E.
Minneapolis, MN 55455
Phone: 612-625-2265

Mega-Resveratrol

http://www.megaresveratrol.net/t
Candlewood Stars Inc.
60 Newtown Rd. (#32)
Danbury CT 06810
USA
Item: MR99P, Mega Resveratrol 99%, 60 grams.

Appendix C

Log Rank Test

Instructions for Long Rank Test

The log rank test compares survival (or mortality) between two experimental conditions. You can perform Log Rank Tests at the following web site: **http://bioinf.wehi.edu.au/software/russell/logrank/.**

The data format for a Log Rank Test differs from what you will most likely use for your trend graphs. First, the data needs to be written in columns; with the first column being the time (days since birth of worms (Table C1).

Table C1. Worms living at each observation day.

Days-elapsed	Strain/Condition-1	Stain/Condition-2
0	30	17
8	30	17
10	28	16
12	26	15
14	26	14
16	26	14
18	21	13
20	17	10
22	12	7
24	7	3
26	0	0

For the Log Rank Test, you will need to convert your data to the accumulated total of dead worms each day. This can be accomplished by the formula (n-x), where n is the total worms in an experiment and x are the number living each time point. Format the data with the running tally of dead worms as follows (Table C2):

Table C2. Worms dead at each observation day (n-x).

Days-elapsed	Strain/Condition-1	Stain/Condition-2
0	0	0
8	0	0
10	2	1
12	4	2
14	4	3
16	4	3
18	9	4
20	13	7
22	18	10
24	23	14
26	30	17

Copy and paste the information into the input box on **http://bioinf.wehi.edu.au/software/russell/logrank/** as follows, making sure the first radio button is selected and then click the Run button (Fig. C1):

You will see a screen similar to that shown in Figure C2. Make Sure all of the fields are completed correctly: Number of individuals is the number of starting worms for the condition. The first line and last line are actually data, not blank spaces or labels. Click Run.

The final screen will be created (Fig. C3). This will provide you with statistical information, and a graph. You need to test all combinations of variables separately in order to determine if there is significance.

Log Rank Test

This page contains a link to a script, designed to perform a log rank test, using the statistical package, R.
Just cut and paste your data into the following box

Enter data here:

```
Days-elapsed    Strain/Condition-1
Stain/Condition-2
0       0       0
8       0       0
10      2       1
12      4       2
14      4       3
16      4       3
18      9       4
20      13      7
22      18      10
24      23      14
26      30      17
```

Column's are separated by a tab ○ , space ○ , comma ○ , or any whitespace ◉ .

◉ The data is a list of time intervals, with the number* of deaths recorded in each group.
○ The data is a list of subjects, with the group and time of death of each subject given.
○ The data has a second, stratified factor. The data is also a list of subjects, with the group, time of death (or time of the end of the experiment if the subject didn't die). Two more columns are required, one giving the second factor and another whether the subject died (0 for survival and 1 for death).

Run Reset

Help can be found here.

*This number can be expressed as
a. the number of deaths for the group in that time period.
b. the percentage of subjects of that group that died in that time period.
c. the number of deaths recorded for the group up until the end of the time period.

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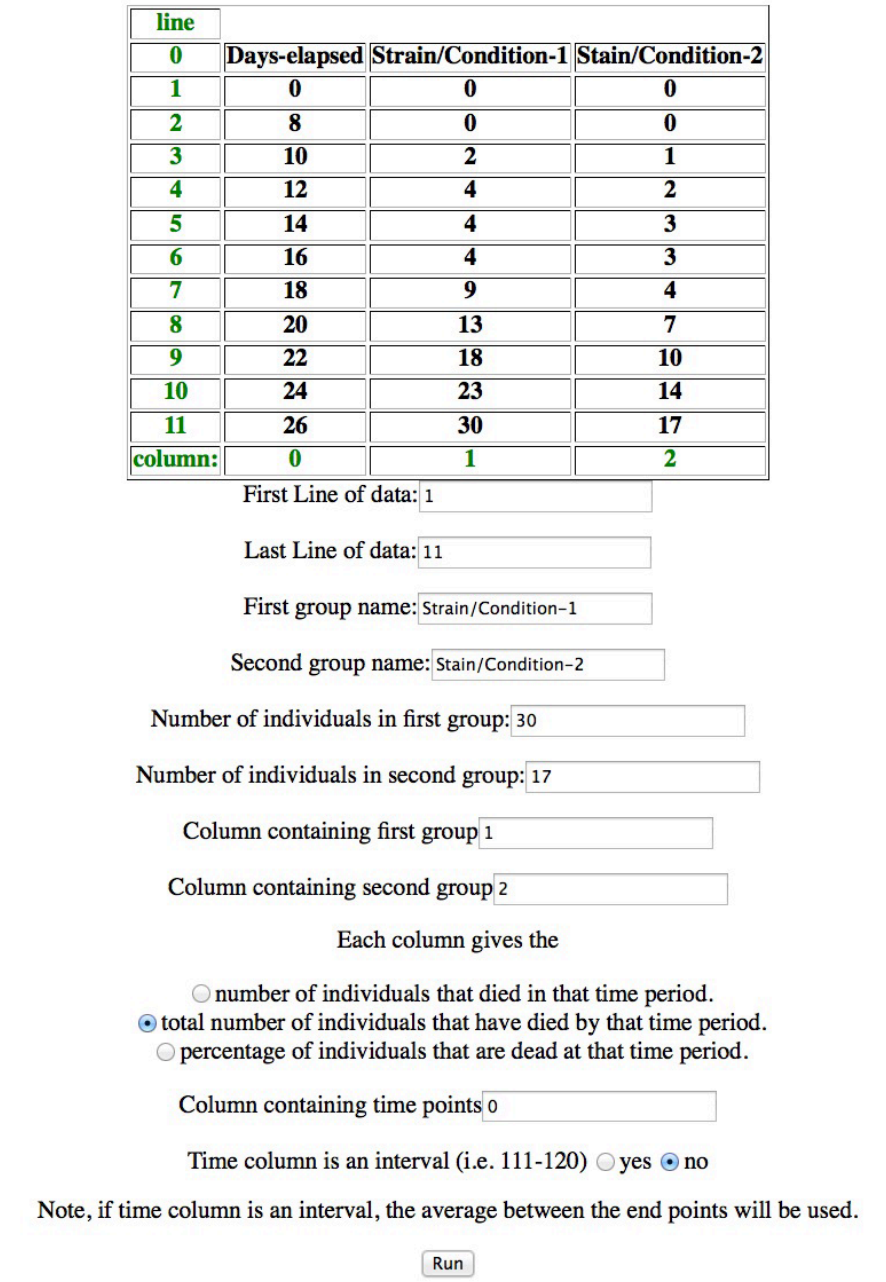
Comments/Questions? Contact Maintainer:Keith Satterley: keith@wehi.edu.au.

Figure C1. Screen view of Log Rank Test at the Walter+Eliza Hall Bioinformatics web site; author: Russell Thomson.



line			
0	Days-elapsed	Strain/Condition-1	Stain/Condition-2
1	0	0	0
2	8	0	0
3	10	2	1
4	12	4	2
5	14	4	3
6	16	4	3
7	18	9	4
8	20	13	7
9	22	18	10
10	24	23	14
11	26	30	17
column:	0	1	2

Figure C2. Second screen shot of Log Rank Test

Strain/Condition-1 vs Stain/Condition-2

Call:

survdiff(formula = Surv(time, censor) ~ group)

	N	Observed	Expected	(O-E)^2/E	(O-E)^2/V
group=0	30	30	30.4	0.00534	0.0245
group=1	17	17	16.6	0.00978	0.0245

Chisq= 0 on 1 degrees of freedom, p= 0.876

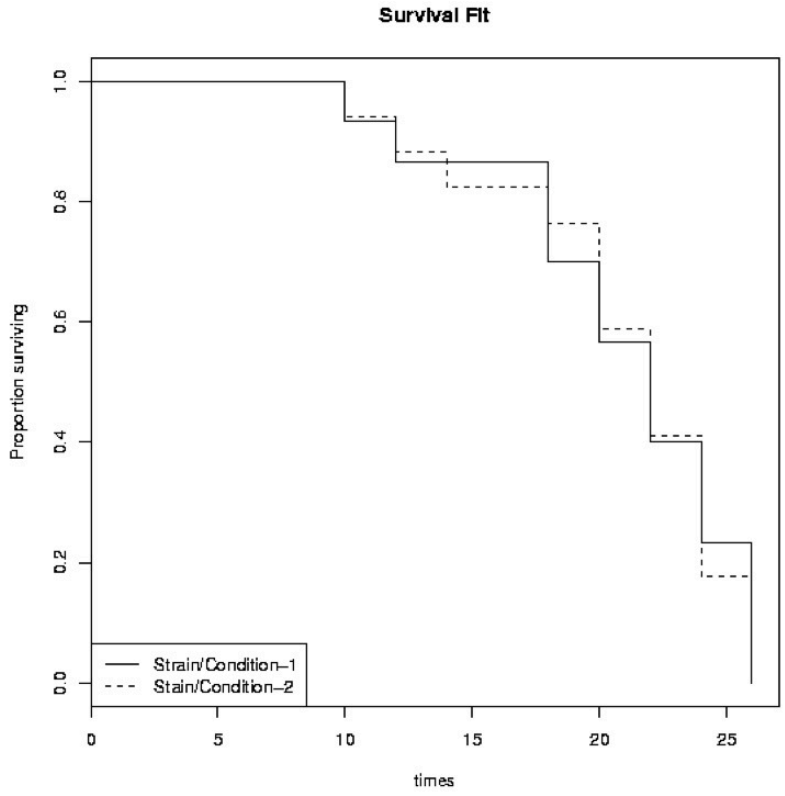


Figure C3. Statistical analysis of two conditions using a log rank test. The graph and p value are computed.

Appendix D

Student Worksheet

Names: _____

Lab Section: _____

C. elegans Longevity

Experimental Proposal Sheet

Write your hypothesis, rationale, and experimental variables on this sheet. You will generally have one control group and up to three experimental groups.

Hypothesis:		
Rationale:		
Experiment	**Strain**	**Condition**
Control	_____	_____
Experimental Group 1	_____	_____
Experimental Group 2	_____	_____
Experimental Group 3	_____	_____

Notes or questions:

About the Author

Pliny A. Smith obtained his B.A. in Biology from Grinnell College in 1992 and his Ph.D. from the University of Missouri in 2001. He has been an assistant professor in the Biology Department of Lake Forest College since 2006. Pliny teaches Developmental Biology, Organism Biology, the Biology of Aging. He uses *C. elegans* both in the classroom as well as to train undergraduates to carry out original research.

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