ONLINE INQUIRY & INVESTIGATION

A Chemosensory Adaptation Module for the Physiology Laboratory from Student-Directed *C. elegans* Research

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Faced with the task of designing a semester's worth of comparative physiology laboratory exercises in my first semester as an assistant professor, I turned to my research and found what I had always suspected–the model organism, *Caenorhabditis elegans*, is as useful in the teaching laboratory as it is in the research laboratory. Since 1974 (Brenner, 1974), research with this common, free-living nematode has yielded numerous significant discoveries in areas ranging from cell signaling and early development to cell death and aging (Table 1). And for good reason; the *C. elegans* system, a.k.a. "the worm," boasts many salient features including being the first multi-cellular organism with a sequenced genome (The *C. elegans* Sequencing Consortium, 1998), a fully described cell lineage (Sulston & Horvitz, 1977; Kimble & Hirsh, 1979; Kimble & White, 1981; Sulston, Schierenberg et al., 1983), and a growing, friendly research community. Many of the attributes that make *C. elegans* an attractive research specimen, such as its relative low cost and simple body plan (Figure 1), also make it an ideal system for the teaching laboratory. In particular, *C. elegans* is well suited to genetics and cell biology teaching applications (Guziewicz, Vitullo et al., 2002; Griffin, McMiller et al., 2003). Similarly, as *C. elegans* physiologists already know, I have found it is well suited to the physiology laboratory. Here, I will describe how students in my junior level college Comparative Physiology course have made use of *C. elegans* in semester-long, student-directed research projects. One of these projects, chemosensory adaptation, can be implemented as a stand alone laboratory module suitable for high school and college students and will be described in detail. Additionally, some general tips to incorporate *C. elegans* into your teaching laboratory will be mentioned throughout this paper.

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Student Directed *C. elegans* Research

As a supplement to several traditional physiology laboratory exercises, including an excellent *ABT* How-To-Do-It on amphibian endocrinology (Heggland, Lawless et al., 2000), I require my students to design and implement physiology research projects involving *C. elegans*. Although I tend to guide the students' projects as little as possible, the level of instructor involvement can be quite variable. By requiring students to write a research proposal prior to initiating their experiments, they can be directed towards realistic projects that are likely to succeed. The only stipulations are that the research investigate some area of physiology discussed in lecture and not reproduce published data or experiments. At the first laboratory meeting, I introduce *C. elegans* as a research organism and ask the students to consider their favorite topic from lecture. From this topic, I help research groups sift through the *C. elegans* literature and formulate a research plan. An important resource is the *C. elegans* Web site, http://elegans.swmed.edu, where students can search all published *C. elegans* papers, as well as unpublished meeting abstracts and articles in the informal *C. elegans* periodical, *The Worm Breeder's Gazette*.

Typically, prior to spring break, the students have solidified a research plan that includes a hypothesis, a detailed experimental design, and identification of the

relevant controls. During the break, I then have time to gather the required reagents and supplies. As the groups begin their experimentation, it is important to regularly check their progress in case experimental design troubleshooting is required. Ultimately, the students are responsible for initiating the experiments, some technical troubleshooting, data collection, and assembly of the data into a research paper. I require that the research papers adhere to a specific journal format, typically one that is readily available for examples, such as *Current Biology*. In this way, they experience the process of converting great research ideas into meaningful publications. In the future, I plan to incorporate peer review by having student research papers reviewed by other students and biology faculty prior to completion of the project. At the end of the semester, I require my students to present their hypothesis, experimental design, data, and conclusions to their colleagues in order to gain experience in oral research presentations and expose all the students to the various research projects.

Table 1. Selected *C. elegans* Research Milestones.

1974	A paper is published that signals the beginning of *C. elegans* as a major research system and includes a description of the use of forward genetics to isolate mutant nematodes with interesting phenotypes (Brenner, 1974).
1976-1987	A complete reconstruction of the *C. elegans* nervous system is produced from serial electron micrographs. This, coupled with the use of genetics, allows a more thorough approach to dissecting the genetic control of behavior and neuronal development (Albertson & Thomson, 1976; White, Southgate et al., 1986; Durbin, 1987).
1977-1983	A series of papers provide a complete description of the invariant embryonic, post-embryonic, and germ line cell lineage. These papers provided the foundation for many studies into the molecular factors controlling cell fate specification (Sulston & Horvitz, 1977; Kimble & Hirsh, 1979; Kimble & White, 1981; Sulston, Schierenberg et al., 1983).
1986	The first identification of genes that control programmed cell death or apoptosis. Apoptosis is a major cellular phenomenon crucial for many aspects of development including the separation of digits in the human hand during gestation (Ellis & Horvitz, 1986).
1994	Description of the use of green fluorescent protein as a reporter for gene expression in living cells, a now nearly ubiquitous reporter gene (Chalfie, Tu et al., 1994).
1998	First description of double stranded RNA mediated interference or RNAi which has become a major tool for gene inactivation in many research systems. Furthermore, research into the mechanisms of RNAi have uncovered the widespread use of short interfering RNAs by the cell to control gene expression (Fire, Xu et al., 1998).
1998	Genome sequence of *C. elegans* published. This was the first description of an entire genome of a multicellular organism. The *C. elegans* genome also served as a trial run for the human genome (The *C. elegans* Sequencing Consortium, 1998).
2002	Sydney Brenner, Robert Horvitz, and John Sulston share the 2002 Nobel Prize in Physiology or Medicine for their research leading to the current understanding of apoptosis (Check, 2002).

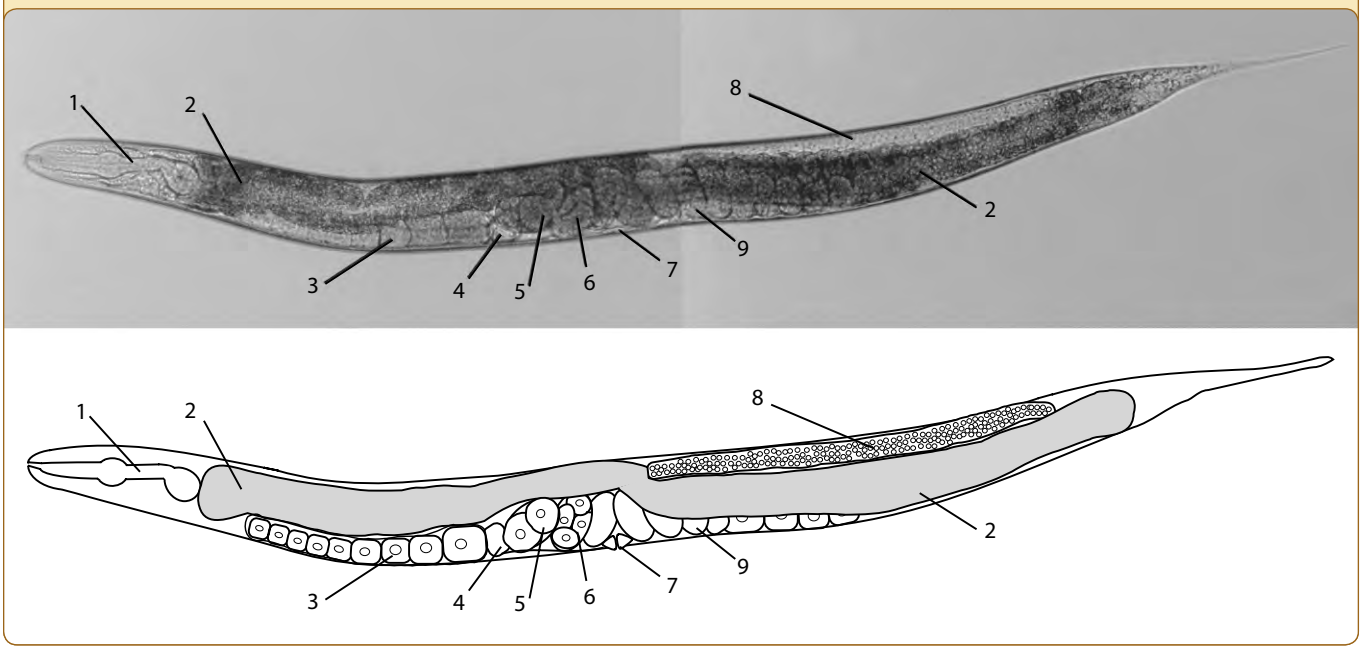
Basic *C. elegans* Husbandry

Care and feeding of *C. elegans* is straightforward and does not require a large time commitment. The most common *wild type* strain, N2, is available for free from the *Caenorhabditis* Genetic Center (http://biosci.umn.edu/CGC/CGChomepage.htm). *C. elegans* is a bacteriotroph, therefore, populations are maintained on Petri dishes containing Nematode Growth Media spread with a lawn of the auxotrophic strain of *E. coli*, OP50, as a food source (Wood, 1988; see Materials & Methods for details). Genetically homogeneous strains, such as N2, can be maintained simply by moving a small cube of agar and nematodes from a plate where the nematodes have consumed all of the bacteria onto the surface of one with a fresh lawn of bacteria (this is referred to as "chunking" by *C. elegans* researchers). Alternatively, individual animals can be transferred to a new plate with a platinum wire that has been flattened on the end with a hammer. After sterilizing in a small alcohol lamp or Bunsen burner, a small dollop of fresh bacteria is swiped onto the bottom of the flat wire end. A light touch with the bacterial swipe will capture a single worm which is released by spreading the bacteria on a new plate. As platinum wire is expensive, small (1-2 cm) lengths can be melted into the end of a Pasteur pipet for use as a handle. At room temperature, the entire life cycle, from fertilization to sexual maturity is completed in approximately 40 hours (Wood, 1988). Therefore, a single hermaphrodite, which can produce up to ~350 progeny, can populate a plate within a few days. Traditionally, worms are grown on 60 mm plates but can also be maintained on 35 mm and 100 mm dishes as well as 12 to 96 well tissue culture plates (the cheaper, uncoated plates work great).

An adult hermaphrodite grows to the length of ~1 mm, therefore, all work must be done under a dissecting stereomicroscope; typically, a good quality microscope with magnification in the range of 10X to 40X. Most teaching

Figure 1. Adult *C. elegans* hermaphrodite (anterior to the left).

Many structures are visible in the transparent nematodes using typical teaching grade dissecting stereomicroscopes. A drawing based upon the image of the adult in the top panel illustrates some of the most prominent tissues and structures. The pharynx (1) contains an anterior pumping bulb and a posterior grinding bulb to move bacteria from the environment into the intestine. In most adults, the intestine (2) is darker than the rest of the animal and takes up a significant portion of the body cavity. Adult hermaphrodites contain two fully functional gonads that begin in the middle, ventral area of the animal near the vulva (the vulva is not visible in the photograph but its approximate position is indicated in both panels – 7) and extend towards the head and tail. Near the ends of the intestine, the two gonad arms turn dorsally and extend back towards the middle of the animal. The portion of the gonads prior to the turn is known as the proximal arm and that after the turn is the distal arm. In this image and drawing, the gut obscures the anterior distal arm and the posterior proximal arm. Oocytes are formed from a population of nuclei in a common, syncytial cytoplasm (8). Nuclei at the distal end of the gonad continually populate the distal arm through mitotic divisions. As nuclei move towards the gonad turn, they enter meiosis in preparation to become oocytes. At the turn, meiotic nuclei obtain a cell membrane and continue to mature as they move towards the proximal end of the gonad (3). Each hermaphrodite typically contains 300-350 sperm housed in two spermatheca (only the anterior spermatheca is visible – 4). Oocytes are fertilized as they pass from the oviduct to the uterus through the spermatheca. Typically, the first embryonic divisions occur in the uterus prior to the embryo's expulsion through the vulva (7). In this animal, a two cell (5), four cell (6), and several multicellular embryos (9) are visible.



stereomicroscopes are only equipped with an incident light source, however, *C. elegans* is best viewed with transmitted light. I have found that replacing an opaque stage with a clear or frosted glass stage and placing the microscope on a light box, such as those used for viewing X-ray films and autoradiographs, is sufficient. A lightly exposed film, such as that of an old sequencing film, can be positioned between the light source and the microscope to enhance the contrast between the nematodes and the media. This eliminates the need to purchase new stereomicroscopes for a few *C. elegans* laboratory modules.

Diverse Physiology Interests Lead to Diverse Research Projects

The most significant advantage to student-directed research in the teaching lab is the increased level of student enthusiasm stemming from their interest and investment in the project. My students respond much more enthusiastically to their own research than to prepared, single-day projects. In addition, because I allow the students to pursue any area of physiology they find interesting, they appreciate the wider diversity of topics than normally presented in the traditional format. For example, in a recent physiology laboratory section of 16 students in four research teams, my students chose a wide variety of projects. One group chose to investigate the effects of antioxidants on the life span of animals exposed to a radiation source. They found that radiation does indeed shorten the *C. elegans* lifespan and that the addition of antioxidants from over-the-counter vitamins appears to partially alleviate the radiation-induced shortening of lifespan. A second group probed the existence of adenosine as a neurotransmitter by assaying the effects of exogenous adenosine and its antagonist, caffeine, on three different mechanosensory responses. Another demonstrated the use of *C. elegans* as a pharmaceutical tool by examining serotonin modulated changes in

behavior in the presence of commonly used serotonin-reuptake inhibitors. Finally, as will be described in detail below, a fourth group examined the time course of chemosensory adaptation to the *C. elegans* chemoattractant, 2-butanone. With each of these projects, because of my requirement for research presentations at the end of the semester, my entire class benefits from the knowledge gained by each research team. Occasionally, students choose research areas, such as aging, that are not normally covered in lecture. Therefore, my class benefits from not only a deeper understanding of a few specialized topics, but also from entirely new areas of their own choosing. For the instructor, the diversity of projects might appear to result in considerable extra work as more background literature must be reviewed to stay on top of the various projects. However, I found the diverse level of research projects to be quite manageable given the relief from preparing and administering more traditional laboratory exercises on a weekly basis. This type of laboratory "preparation" also allows the instructor to stay current and enthusiastic by providing protected time to read the scientific literature.

Chemosensory Adaptation to 2-Butanone

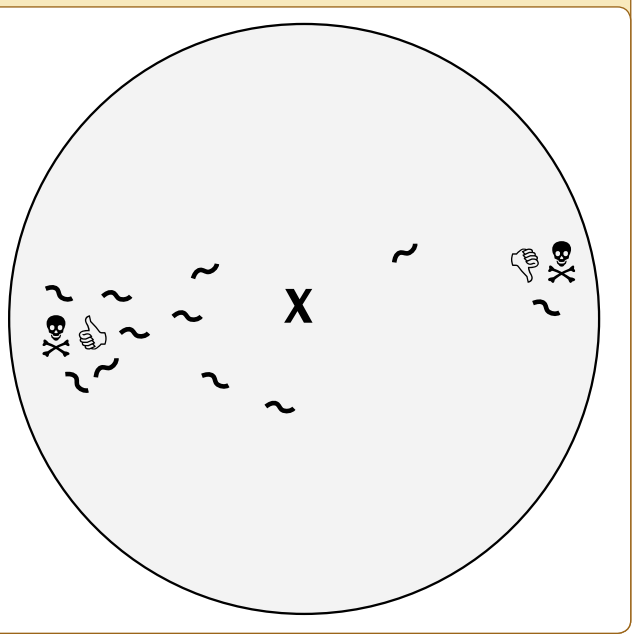
Sensory adaptation is a readily observed phenomenon and most students experience this physiological response daily. Adaptation, or the diminished perception of stimuli in the environment, is an important modulator of neural activity and allows animals to focus on changes in the environment rather than static stimuli. Examples of sensory adaptation that help students understand the concept are the diminished sensation of wearing clothes when sitting still, adapting to cold water when swimming, and the inability to smell cologne, perfume, or body odor after a period of time.

C. elegans chemosensory assays (Figure 2) provide a means to quantify adaptation as well as examine its exposure requirements. Basing their experiments primarily upon the research of Cori Bargmann at UCSF, three physiology students chose to investigate the time course of chemosensory adaptation in *C. elegans*. They hypothesized that exposure to a chemoattractant would result in adaptation and a diminished attraction to a given odorant as a function of time. They predicted that short exposures would result in little or no adaptation, and longer exposures in a stronger or more complete adaptation to known odorants. From a review of the literature as well as *C. elegans* meeting abstracts, three previously identified volatile attractants were selected primarily for their relative safety and availability—isoamyl alcohol, 2-butanone, and pyridine (Bargmann, Hartwieg et al., 1993).

In order to test their hypothesis, the students first ranked these three odorants in terms of attractiveness by using a chemosensory assay (Ward, 1973; Bargmann & Mori, 1997). This assay allows quantification of the attractiveness of an odorant in comparison to a control or additional odorant. Nematodes were placed onto the center of a standard *C. elegans* 100 mm culture dish that lacked the bacterial food source. On opposite sides of the dishes were spots of odorants in addition to a paralyzing agent (such as 1% sodium azide). After one hour, each odorant's attractiveness (or repulsion) was to be measured by the number of animals found near the odorant spots. The paralyzing agent effectively records an individual worm's first preference by preventing the worms from subsequently moving to another area of the plate after adapting to the preferred odorant. The students were interested in obtaining a ranking of chemo-attractiveness and therefore created a tournament schedule (they were all student athletes on the basketball team) pitting odorant vs. odorant. The results of this testing showed clearly that *C. elegans* prefers 2-butanone, followed by isoamyl alcohol, and finally pyridine (Figure 3). Alternatively, a rank ordering could also be obtained by placing all three odorants on a single chemosensory assay plate.

Figure 2. *C. elegans* chemosensory assay.

Wild type or mutant *C. elegans* adults that are placed onto the center (X) of a standard NGM plate will migrate towards a chemoattractant (👍) or away from a chemorepellant (👎). To quantify chemo-attractiveness, a small quantity of odorant or control is spotted onto the media along with an anesthetic such as 1% sodium azide (☠). Animals will migrate towards the preferred odorant and become paralyzed, facilitating a quantitative test for the preference of the animals towards two chemicals or vs. a control substance.



As a stand-alone laboratory module, this portion of the experiment is sufficient to be used as the first of a multi-lab period set of experiments. The assay is quite dramatic, especially when 40-50 animals are used on each plate. To accommodate and handle this large number of animals, it is useful to wash the animals from standard growth plate using an isotonic solution such as M9 (recipe in Materials

& Methods). When placed on ice for 10-15 minutes, the nematodes will settle to the bottom of a culture tube, allowing the concentration of many animals. A small drop of concentrated animals can then be placed on the center of the assay plate and will contain sufficient numbers. To allow the worms to escape the liquid drop on the plate, use either slightly dehydrated dishes (which can be made by simply storing the plates upright for several days in a well ventilated area) or remove the liquid with a drawn Pasteur pipet.

Care should be taken when handling the anesthetic (sodium azide is a toxic compound) and odorants. It is best to apply the odorants in a fume hood as they are quite volatile and prolonged opening of the bottles can saturate a work area foiling the experiment. We have applied the sodium azide and odorants to identical locations within a few minutes of each other without complications.

After ranking, the odorants are retested using the same protocol with animals that have been exposed to the odorants during an adaptation period. To adapt an entire population of animals to an odorant, simply place a small volume of the chemical in the lid and incubate the population-containing plate upside down on top of the lid for a specified time. My students found that, as predicted, longer adaptation times resulted in chemosensory adaptation to the odorant. This adaptation is evident in the transition of preference from 2-butanone to isoamyl alcohol when adapted to 2-butanone (Figure 4).

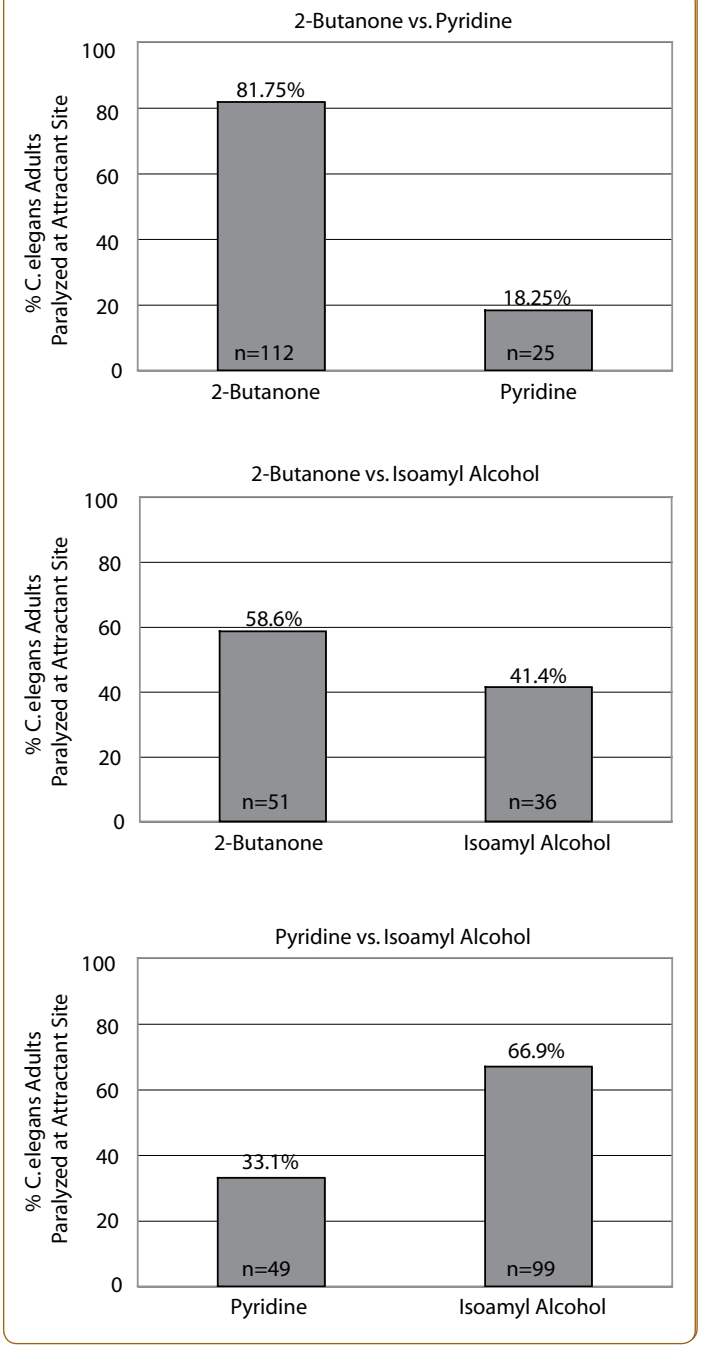
The phenomenon of chemosensory adaptation is not perfectly understood at the cellular level. However, it is clear that like many animals, *C. elegans* becomes less sensitized to a particular odorant when exposed to that odorant for a prolonged period in the absence of food. Further, this desensitization, or adaptation, does not affect the animal's ability to detect another odorant even when that odorant is detected by the same olfactory neuron (Bargmann & Mori, 1997). Thus, chemosensory desensitization to particular chemicals likely involves changes at the subcellular level such as a decrease in odorant-specific receptors or down-regulation of the second messenger signal propagation resulting from cell-surface receptor activation.

Chemosensation & Inquiry-Based Learning

While this is a fairly straightforward demonstration of chemosensory adaptation, the level of difficulty and emphasis on inquiry-based learning can be increased by limiting the amount of background material provided to the students. As suggested above, chemosensory preference within populations of animals could be demonstrated with only a single assay plate and two odorants. Such an experiment would be appropriate for a single three-hour laboratory period if performed by the students. As a simple demonstration performed by the instructor, this experiment could run the course of a single lecture period. For increased rigor, multiple rank orderings and adaptation experiments would allow the inclusion of statistics to further validate the findings. For example, two to three

Figure 3. Pair-wise tests demonstrate that wild type *C. elegans* adults prefer 2-butanone over both isoamyl alcohol and pyridine.

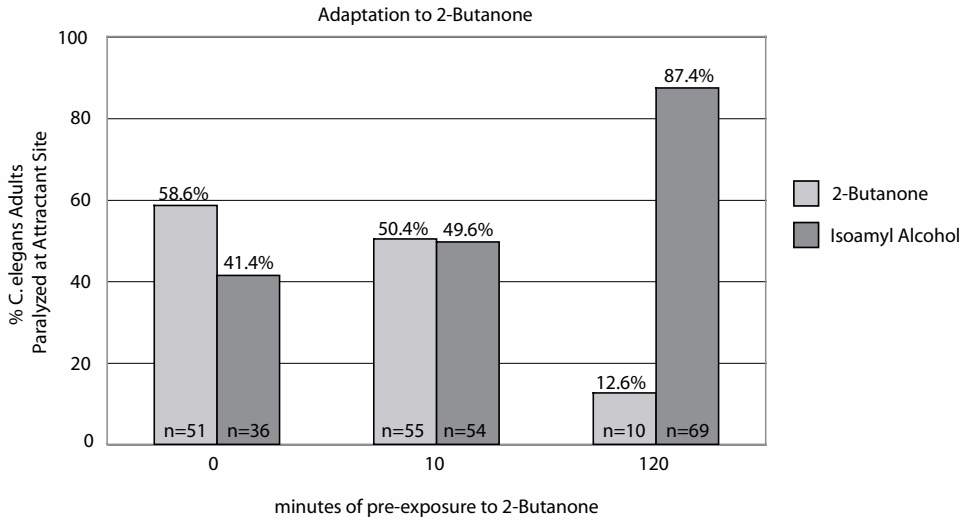
Using the chemosensory assay described in Figure 2, we compared three odorants in pair wise tests for *C. elegans* preference (A-C). Naïve animals, with no previous exposure to the chemicals, preferred 2-butanone over isoamyl alcohol and were attracted to pyridine the least. n = number of animals



laboratory periods could be devoted to obtaining several rank ordering trials that would yield data in the form of the average ± the standard deviation for three or more populations of animals. For a semester-long project, students could search for new odorants sensed by *C. elegans*. Additionally, there are many genes known to be involved in chemosensory adaptation such as *osm-9, adp-1* (Colbert & Bargmann, 1995), and *tax-6* (Dusenbery, 1976), all of which have been altered to produce chemosensory defective mutants and are available from the Caenorhabditis Genetic Center (CGC). Students may also wish to test other known chemoattractants and repellents or simply test a battery of chemicals for chemosensation by *C. elegans*. An excellent discussion of chemosensation, as well as the equally fascinating behavior of thermosensation, and a table of odorants can be found in the book *C. elegans II* (Bargmann & Mori, 1997). Each semester that I teach comparative physiology, a student group picks chemosensation as a research topic. I have found that allowing students to design their own experiments has resulted in a wide variety of chemosensory exploration.

Figure 4. Adaptation to 2-butanone changes *C. elegans* preference to a lesser chemoattractant.

When pre-exposed to 2-butanone, *C. elegans* becomes adapted to the presence of the odorant. This adaptation is evident by their increasing preference for isoamyl alcohol, which is normally a lesser preferred chemoattractant, with increased pre-exposure time to 2-butanone. n = number of animals



C. elegans in the Teaching Laboratory

Because of its role as an important model research organism, data from the *C. elegans* system is becoming more common in biology texts. Incorporating these nematodes into the laboratory will help students get a better grasp of the material. This laboratory exercise is one way that instructors at many educational levels can become acquainted with the system. Also, due to the rich research history with *C. elegans* chemosensation, this module can be adapted for use by already competent *C. elegans* researchers and educators. As either a single exercise demonstrating chemosensation or as a semester-long research project exploring the genetics of the adaptation, the *C. elegans* chemosensory system will hopefully add a new dimension to your teaching laboratory.

Materials & Methods

Care and Feeding of *C. elegans*

Populations of *C. elegans* (available from the CGC) containing all life stages can be easily maintained on nematode growth agar (NGM) which is prepared by adding:

- 3 g NaCl
- 2.5 g peptone
- 17 g agar

to

- 975 ml dH_2O.

Autoclave

After cooling to 55° C, use sterile technique to add:

- 1 ml cholesterol (5 mg/ml in ethanol)
- 1 ml of 1 M $CaCl_2$
- 1 ml of 1 M $MgSO_4$
- 25 ml of 1 M potassium phosphate (pH 6)

and pour into sterile, vented Petri dishes (Wood, 1988).

Another, slightly easier, recipe (called NGM-lite) is prepared by mixing:

- 2 g NaCl
- 4 g bactotryptone
- 3 g KH_2PO_4
- 0.5 g K_2HPO_4

- 1 ml of 5 mg/ml cholesterol in ethanol
- 17 g agar

  in
- 1 liter dH_2O.

Autoclave, and pour into dishes (adapted from Eric J. Lambie's article in the *Worm Breeder's Gazette* issue 13 [5], February 1, 1995).

Bacterial lawns are grown by spreading a small volume of a saturated culture of the OP50 strain of *E. coli* (available from the CGC) on the plates and incubating overnight at 37° C. M9 solution for washing nematodes off the plates is a sterilized solution of :

- 3 g KH_2PO_4
- 6 g $NaHPO_4$
- 5 g NaCl
- 1 ml of 1 M $MgSO_4$

  in
- 1 liter of dH_2O (Wood, 1988).

Chemosensory Assay Plates

Chemosensory assay plates are prepared by spotting 2 µl 100% odorant, along with 2 µl 1% sodium azide on either sides of a Nematode Growth Media (NGM) culture dish that lacks the spread bacterial lawn. An alternative anesthetic is a mixture of 0.1% tricaine and 0.01% tetramisole or 1% 1-Phenoxy-2-propanol (Hall, 1995). Care should be taken with many anesthetics as they are often known toxins. Odorants should be spotted in a fume hood to reduce exposure of students and nematode populations to the volatile compounds. Chemosensory adaptation can be obtained by placing a 100 µl of odorant/control onto the lid of an inverted NGM plate containing an actively growing nematode population for a specified amount of time.

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