

## Using the *Wolbachia* Bacterial Symbiont to Teach Inquiry-Based Science: A High School Laboratory Series

SETH R. BORDENSTEIN, CHRISTINE BROTHERS,  
GEORGE WOLFE, MICHELE BAHR,  
ROBERT L. MINCKLEY, MICHAEL E. CLARK,  
JENNIFER J. WERNEGREEN,  
SARAH R. BORDENSTEIN, WILLIAM S.  
REZNIKOFF, JOHN H. WERREN



Image provided by Michael Clark.

### ABSTRACT

*Inquiry, discovery, and technology are key pillars in improving science education. We present an inquiry-based lab project using the worldwide symbiosis between the bacterium Wolbachia and invertebrates. This endeavor, called "Discover the Microbes Within! The Wolbachia Project," has the following goals: (1) involve students in grade 7 through college in a nationwide effort to collect new genetic information on Wolbachia; (2) provide curriculum, protocols, and training to enhance teachers' ability to lead students through the project; and (3) increase students' understanding of biodiversity, evolution, symbiosis, cell biology, molecular biology, and bioinformatics.*

**Key Words:** Symbiosis; microbiology; DNA; biotechnology; Wolbachia.

Humans consist of approximately 10% human cells and 90% prokaryotic cells, yet the idea of studying the varied relationships between eukaryotic hosts and prokaryotic symbionts is largely ignored in introductory biology classes. "Discover the Microbes Within! The *Wolbachia* Project" is an innovative lab series that gives students the opportunity to make original scientific discoveries about invertebrate endosymbionts while learning integrative approaches in biodiversity, evolution, cell biology, molecular biology, and bioinformatics. The project utilizes the widespread symbiont *Wolbachia*, inherited intracellular bacteria that live within the cells of the reproductive tracts of ~66% of arthropod species (Hilgenboecker et al., 2008). One arthropod subgroup, the insects, comprise ~85% of all animal species, which makes *Wolbachia* perhaps the most common bacterial endosymbiont in the biosphere. Estimates place these bacteria in millions of arthropod species.

*Wolbachia* are primarily passed from mother to offspring through the cytoplasm of the egg (Figure 1) but can also be horizontally transmitted between arthropods. *Wolbachia* are commonly referred to as "reproductive parasites" because they manipulate sexual reproduction in their hosts in four ways (Werren et al., 2008). *Wolbachia* can kill infected males outright (male killing), cause male offspring to develop into females (feminization), cause females to reproduce asexually or clonally (parthenogenesis), or kill the offspring of an uninfected female when its mate is infected (cytoplasmic incompatibility). All these strategies increase the number of *Wolbachia*-infected females, thereby increasing parasite transmission. *Wolbachia* was first discovered in the 1920s, but

its significance to insect speciation (Bordenstein, 2003), evolution, and ecology (Stouthamer et al., 1999; Werren et al., 2008) and to human health (Taylor, 2002; Sinkins & Gould, 2006; McMeniman et al., 2009) was little appreciated until recently.

In addition to insects, *Wolbachia* are also known to infect filarial nematodes, terrestrial crustaceans (isopods), mites, scorpions, and spiders. *Wolbachia* infections play an important role in filarial nematodes that cause the human diseases onchocerciasis ("river blindness") and elephantiasis (Taylor et al., 2005). An electron micrograph of *Wolbachia* in filarial nematodes is shown in Figure 2. Treatment with antibiotics eliminates the *Wolbachia* from these disease-causing worms, thereby killing or sterilizing the worms. *Wolbachia* is also a potential biological control agent or genetic vector for spreading desirable genetic modifications in insects (Sinkins & Gould, 2006; McMeniman et al., 2009).

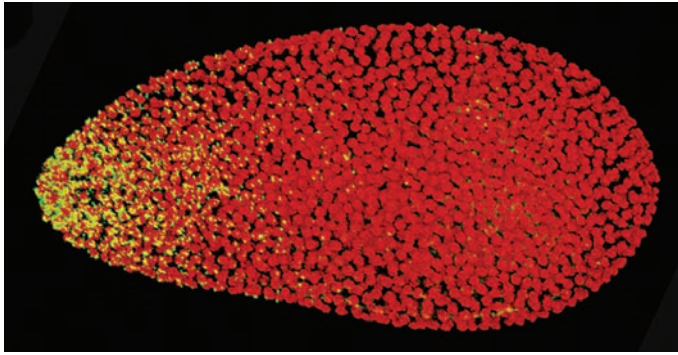
*Wolbachia* biology is poised to initiate curiosity and inquiry in the classroom. The inquiry for this project can be introduced in many ways, by asking questions such as "Why would organisms want to live inside the cells of another? What mechanisms have the bacteria evolved to spread through host insects? What can we learn from *Wolbachia* biology to control the spread of insect-borne West Nile virus or agricultural pests? What does this symbiosis tell us about the nature of our own cells?" All these questions have exciting answers from this single system. Notably, the

*Wolbachia* scientific community isn't big enough by itself to adequately sample the world's arthropod and nematode populations for this infection. Students are potentially the biggest assets in helping scientists study these important topics.

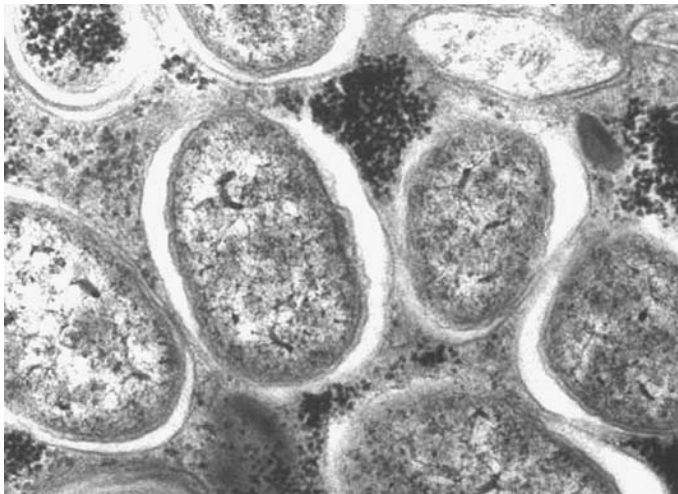
*Upon completion of the labs, students can submit any positive Wolbachia samples to the MBL for free DNA sequencing.*

### ○ Discover the Microbes Within! The *Wolbachia* Project

The lab exercises, originally conceived by John H. Werren, were developed in 2004 as part of a National Science Foundation Frontiers in Integrative Biological Research (FIBR) award. The goals of this award were to integrate genetic, cell biology, ecological, and environmental aspects of *Wolbachia* biology. In this same spirit, a series of integrated laboratory exercises were designed to use *Wolbachia* to teach students about biodiversity, ecology, entomology, genetics, cell biology,



**Figure 1.** Insect embryo, showing the dividing insect nuclei (red) and the maternally transmitted *Wolbachia* bacteria (yellow dots) localized to the pointed end of a wasp embryo. (Image provided by Michael Clark.)



**Figure 2.** *Wolbachia* bacteria in the filarial nematode *Onchocerca volvulus*, the causative agent of human river blindness. (Image reproduced from the Encyclopedia of Life, Mark Taylor.)

molecular biology, and bioinformatics (Figure 3). Additional support from the NASA Astrobiology Institute and the Howard Hughes Medical Institute has promoted a national expansion of the lab series to thousands of high school students. These laboratories have also been adapted for use in college undergraduate teaching curricula. In addition, teacher enhancement workshops have been taught annually at the Marine Biological Laboratory (MBL), the University of Rochester, and the Cornell Institute for Biology Teachers.

This lab series addresses *National Science Education Standards* related to science as inquiry, the molecular basis of heredity, biological evolution, interdependence of organisms, science and technology, and science as a human endeavor. The labs can be taught individually throughout the year or as a 2-week unit in approximately eight 1-hour class periods. The *Wolbachia* Project involves students in authentic scientific research that generates data on the infection rate and geographic distribution of *Wolbachia*. Upon completion of the labs, students can submit any positive *Wolbachia* samples to the MBL for free DNA sequencing and publish and compare their *Wolbachia* sequences with those in a national database (<http://discover.mbl.edu/search.php>). As an extension to the labs, students can also determine the frequency of a bacteriophage that infects *Wolbachia* by performing a second polymerase chain reaction (PCR) using phage-specific primers (Bordenstein & Wernegreen, 2004). Through their participation, students contribute to the scientific community's understanding of this important microbial symbiosis.

## ○ Learning Goals & Objectives

The students

- use an online taxonomic key to identify insects they have collected.
- extract genomic DNA from insects and *Wolbachia*.
- amplify a gene fragment using PCR.
- determine the presence or absence of *Wolbachia* and insect genes in the PCR product using gel electrophoresis.
- identify an unknown nucleotide sequence from an insect endosymbiont using the National Center for Biotechnology Information (NCBI) search tool BLAST.



**Figure 3.** Flow chart of the labs in "Discover the Microbes Within! The *Wolbachia* Project." (Image provided by Michael Clark.)

- publish DNA sequence data from insects that test positive for *Wolbachia*.

## ○ Preparation

The protocols for the lab series are summarized below. More detailed and reproducible protocols can be found at <http://discover.mbl.edu>.

The students will embark on their *Wolbachia* expedition by determining which insects to collect and study. They should collect at least two individuals of one to three morphologically different types of insects. They can work in pairs or small groups to identify specimens from the local community. Should they focus on different habitats, locations, temperatures, altitudes, specific lifestyles, or plant associations? Collecting their own insects will give the students immediate ownership of the research. Collected insects should be preserved in vials containing 95% ethanol, labeled with the students' names, collection sites, and dates, and placed in a freezer for later identification. Smaller species are easier to preserve. On the basis of worldwide sampling efforts, the students can expect a positive *Wolbachia* diagnosis in one out of five insects.

Positive (*Wolbachia*-infected) and negative (*Wolbachia*-uninfected) insect controls, *Wolbachia* DNA, and primers for PCR amplification of insect and *Wolbachia* genes are provided at no charge. Registration to obtain primers and controls is available at <http://discover.mbl.edu/register.php>. Allow at least 1 month prior to beginning the experiment to schedule shipment. It is imperative that all labs incorporate sterile technique to prevent contamination and produce trustworthy, reproducible results. Each workbench should be washed with 70% ethanol prior to experiments, and gloves and safety goggles should be worn at all times. Pestles and pipette tips should be discarded after each use and never come into contact with more than one sample or reagent.

## ○ Insect Identification

### Materials (per work station)

- Arthropods preserved in 95% ethanol
- Forceps
- Dissecting probe
- 3 Petri dishes
- Eyedropper or transfer pipette
- 95% ethanol
- 6 glass vials or similar containers
- 3 microcentrifuge tubes
- Sharpie marker, extra-fine point
- Labeling tape
- Digital camera or colored pencils
- Dissecting microscope (optional)
- Computer with Internet access
- Freezer

Using forceps, submerge insects in ethanol-filled Petri dishes. Wash the insects with ethanol and keep them submerged throughout the procedure. Select three individuals from each morphospecies (i.e., individuals with similar morphology that are probably in the same species) and, if possible, select three different morphospecies to be analyzed for *Wolbachia*; one individual will be used for molecular analyses and the other(s) will be stored as vouchers (i.e., copies of the specimen). Document each morphospecies with a digital camera or by carefully drawing with colored pencils, and note proper identification using the Discover Life

online tool for insects ([http://pick4.pick.uga.edu/mp/20q?guide=Insect\\_orders](http://pick4.pick.uga.edu/mp/20q?guide=Insect_orders)). If you are using arthropods other than insects, such as arachnids, isopods, et cetera, you will need to use a separate key.

Transfer one of each morphospecies into a microcentrifuge tube and the voucher species into a vial filled with ethanol. Label with students' initials, date, insect order, and specimen number. If the insects are large, use a scalpel to remove and place a small piece of the abdomen into the microcentrifuge tube (~2 mm<sup>2</sup>; *Wolbachia* are concentrated in the reproductive organs in the abdomen). For small insects, place the entire body in the tube. Completely cover the insects with ethanol and freeze.

## ○ DNA Isolation

### Materials (per work station)

- 3 morphospecies (from previous activity)
- +/- controls (from MBL)
- Ten 1.5-mL microcentrifuge tubes
- Sharpie marker, extra-fine point
- Forceps
- Kimwipe or tissue
- 5 microtube pestles
- DNeasy Blood and Tissue Kit (Qiagen, no. 69504; individual aliquots for each station)
- 95% ethanol
- Vortex mixer
- Microcentrifuge (to 13,000 rpm; one for the class)
- P20, P200, P1000 pipettes and tips
- 70°C Water bath (with float racks) or incubator (one for the class)
- 4°C refrigerator (one for the class)

### Cell Lysis

Figure 4 shows the materials for DNA isolation and amplification (PCR). Label five microcentrifuge tubes with students' initials and sample ID. Place 180 µL of ATL buffer into each tube. Blot each ethanol-preserved



**Figure 4.** Materials needed for DNA isolation and amplification labs. (Image provided by Christine Brothers.)

insect dry and carefully transfer them to the ATL. Thoroughly macerate the insect in tube one with a microtube pestle, working as quickly as possible to avoid DNA degradation. When the insect is macerated, quickly add 20  $\mu\text{L}$  of proteinase K to destroy DNase enzymes and then add 200  $\mu\text{L}$  of buffer AL to break open the cells. Repeat the process with the other four insects. Once the proteinase K is added, the DNA will be relatively stable. Mix by vortexing for 10 seconds. Incubate tubes for at least 10 minutes at 70°C (or overnight at 56°C) in a water bath or incubator. After incubation, add 200  $\mu\text{L}$  of ethanol to each tube in order to precipitate DNA. Vortex or invert as before. Samples can be stored in a 4°C refrigerator overnight.

### Cellular Debris Removal

Fit five DNeasy spin columns into the five collection tubes provided with the columns and label both with sample ID and students' initials. Pipette the liquid from each tube with the insect DNA into the appropriate spin column. Centrifuge all tubes for 1 minute at 8000 rpm to collect the DNA in the filters of the spin columns, and discard the flow-through. Place the spin columns back into their respective collection tubes, add 500  $\mu\text{L}$  of buffer AW1 (wash 1), and centrifuge for 1 minute at 8000 rpm. Again, discard the flow-through and place the spin columns back into their respective collection tubes. Add 500  $\mu\text{L}$  of buffer AW2 (wash 2) to each of the five tubes and centrifuge for 3 minutes at 13,000 rpm. If ethanol remains in the columns, air dry them for 5 minutes. Transfer each spin column to a new microcentrifuge tube labeled with sample ID and students' initials.

### DNA Elution

Pipette 100  $\mu\text{L}$  of buffer AE (elution buffer) directly onto the membrane of each spin column. This is an elution buffer that rinses the DNA off the spin-column filter. Incubate at room temperature for 1 minute and then centrifuge at 8000 rpm for 1 minute. Discard the spin column and store the tube containing DNA at 4°C.

## ○ DNA Amplification (PCR)

### Materials (per work station)

- Sample DNAs (generated in previous activity)
- Control DNAs (from MBL)
- 6 PureTaq PCR Ready tubes (GE Healthcare, no. 27-9559-01)
- Microcentrifuge tube rack
- 20  $\mu\text{L}$  each of Wspec forward and reverse primers (5  $\mu\text{M}$ , from MBL)
- 20  $\mu\text{L}$  each of CO1 forward and reverse primers (5  $\mu\text{M}$ , from MBL)
- 200  $\mu\text{L}$  reagent grade  $\text{H}_2\text{O}$
- Thermal cycler (for class)
- Freezer

### Overview

The students will amplify a fragment of DNA that codes for a specific variant of the *Wolbachia* small subunit ribosomal RNA (16S rRNA) gene. They will also amplify a fragment of DNA that codes for the cytochrome oxidase I protein in animal mitochondria. Thus, if DNA isolation and PCR were successful, students will get a band for amplified mitochondrial DNA from their insect on their gel even if the insect did not harbor *Wolbachia*.

### PCR

Collect six PCR Ready tubes. Each of these contains a preformulated pellet containing Taq polymerase,  $\text{MgCl}_2$ , buffer, and dNTPs. Label each

tube with sample ID and students' initials. Add the following materials to each tube: 15  $\mu\text{L}$  sterile distilled water, 2  $\mu\text{L}$  W-spec forward primer, 2  $\mu\text{L}$  W-spec reverse primer, 2  $\mu\text{L}$  CO1 forward primer, and 2  $\mu\text{L}$  CO1 reverse primer. Finally, add 2  $\mu\text{L}$  DNA template from each insect sample and the control to their corresponding tubes. Cap and tap the bottom of each tube to mix. Place all tubes into a thermal cycler programmed with the following settings:

- 2 minutes at 94°C
- 30 seconds at 94°C; 45 seconds at 55°C; 1 minute at 72°C (30 cycles)
- 10 minutes at 72°C

When the program has ended, store the tubes in a freezer.

## ○ DNA Analysis (Gel Electrophoresis)

### Materials (per work station)

- PCR product (from previous activity)
- Agarose powder
- TAE buffer
- 500 mL Erlenmeyer flask
- Oven mitt or tongs (for class)
- Gel casting tray and combs
- Labeling or masking tape
- Electrophoresis box and power supply
- Parafilm or wax paper
- 6 $\times$  loading dye
- DNA ladder
- QUIKView DNA Stain (Ward's, no. 38 V 9014)
- Hot plate or water bath at 55°C
- Staining trays (for class)
- Optional: Ziploc bag, ice pack, and Styrofoam box for shipping

### Loading & Running the Gel

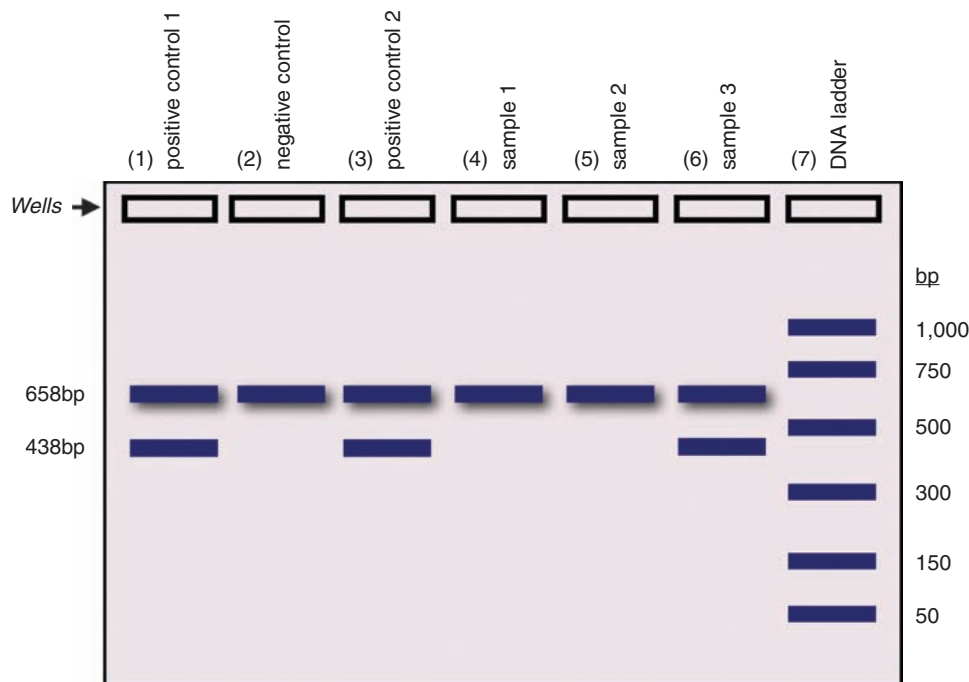
Prepare a 2% agarose gel. Record the order in which each sample will be loaded into the gel. Add 10  $\mu\text{L}$  of each PCR reaction to 2  $\mu\text{L}$  of 6 $\times$  loading dye on a piece of parafilm or wax paper and mix by pipetting up and down. Transfer this mixture into the designated well in the gel. The remaining PCR product should be frozen in case there is a need for future sequencing. Pipette 10  $\mu\text{L}$  of DNA ladder into one well of each row on the gel. Place the lid on the gel box, connect the electrodes, and run at 100 volts until the loading dye approaches the end of the gel.

### Gel Staining

Place the gel into the staining dish. Cover with warmed (50–55°C) DNA stain. Allow the gel to stain until dark blue (at least 30 minutes) and remove the stain, which can be reused for additional gels. Rinse the gel in the staining tray with warm water to remove excess stain. The gel will become lighter, leaving only dark blue DNA bands, and may need to be destained overnight. View the gel against a light box or bright surface (Figure 5). Record and discuss the data while the gel is fresh, because very light bands may be difficult to see over time.

## ○ DNA Sequencing (Optional)

All samples that show a band on the gel for both the insect and *Wolbachia* can be sent to the MBL for sequencing. Shipping instructions will be included



**Figure 5.** Schematic of a gel, showing the PCR products amplified from controls and samples infected and uninfected with *Wolbachia*. The mitochondrial CO1 product runs at 658 base pairs (bp), whereas the *Wolbachia* product runs at 438 bp. The DNA ladder is used to size the PCR products. Sample 3 indicates a *Wolbachia*-infected insect. (Image provided by Michael Clark.)

when the primers and controls are sent. One of the most exciting outcomes of sequencing is that students may generate a sequence new to science. This is an effective motivator because it is publishable online at the project's Web site and is accessible by students, teachers, families, and scientists.

## ○ DNA Sequence Analysis (Bioinformatics)

### Materials (per work station)

- Computer with Internet access

This exercise is designed to introduce the student to how biologists organize and analyze genetic sequence information with bioinformatics. It teaches students to use the taxonomy functions of NCBI and simple BLAST exercises to search in a Google-like manner for a match to their *Wolbachia* DNA sequence. Go to the NCBI homepage (<http://www.ncbi.nlm.nih.gov>) and select "BLAST" in the right menu bar under "Popular Resources." Select "nucleotide blast" under the "Basic BLAST" category and input the *Wolbachia* DNA in the blank box at the top under "Enter Query Sequence." Click on the circle for "Others (nr etc.)" under the section "Choose Search Set." Page down and select "BLAST" at the end of the page. Wait for the results page to automatically launch. The best match to your *Wolbachia* sequence will be reported at the top of the "Descriptions" list, including the "Max Ident" (i.e., similarity) between the input sequence and the best match in the database.

## ○ Assessment

The project has two levels of evaluation. First, the students can be given a pre- and posttest, designed by a professional evaluation agency, before and after the lab series to assess learning outcomes of major biological concepts and student interest in science. Second, the students can present their research in a formal lab report, including research question, hypothesis,

introduction, materials, procedure, data charts, photos or insect drawings, and conclusions. Just as scientists would in a research laboratory, it is important for the students to revisit their initial collection scheme and communicate data within the context of a hypothesis. The students can also be asked to write a summary reflecting on their experience during the lab series. This quote from a student's lab report is typical of what they write:

This lab made the science of DNA less of a mystery and allowed us to take knowledge we learned in the classroom and apply it in the lab. There was an actual scientific purpose to this lab which made it more significant and worthwhile to perform. It was exciting to participate in a real scientific study and provide information that might be useful to the scientific community. It was a cutting-edge lab with techniques many undergraduates don't even get to do. I enjoyed it most out of all the labs performed this year.

## ○ Teacher Resources

All lab instructions, slide presentations introducing each lab, and learning standards addressed, as well as classroom resources and projects contributed by teachers, are available from the project's Web site. Free 3-day training workshops are held each April at the MBL; however, it is not necessary to attend a workshop in order to participate. Thermal cyclers are available for a designated time to schools that do not have this piece of equipment. Six-week paid summer research fellowships to further investigate *Wolbachia* at a local research institution are also available to teacher-student pairs who have conducted the project in their classroom. The research fellowships aim to dissolve perceived disconnects between students and scientists and influence some participants to consider life-science majors in college.

## ○ Online Resources

Discover the Microbes Within! The *Wolbachia* Project: <http://discover.mbl.edu>

Precollege science education labs, videos, and resources for a discovery-based lab series on *Wolbachia*

Discover the Microbes Within! The *Wolbachia* Project at the University of Rochester: <http://www.rochester.edu/college/bio/labs/WerrenLab/WerrenLab-WolbachiaWorkshops.html>

Protocols and PowerPoint lectures

*Wolbachia pipientis*: <http://www.eol.org/taxa/17123196>

An exemplar species page at the Encyclopedia of Life

*Wolbachia*, A Heritable Pandemic:

<http://serc.carleton.edu/microbelife/topics/wolbachia/index.html>

Online resources, news releases, primary literature, WebQuest, and educational modules

## ○ Acknowledgments

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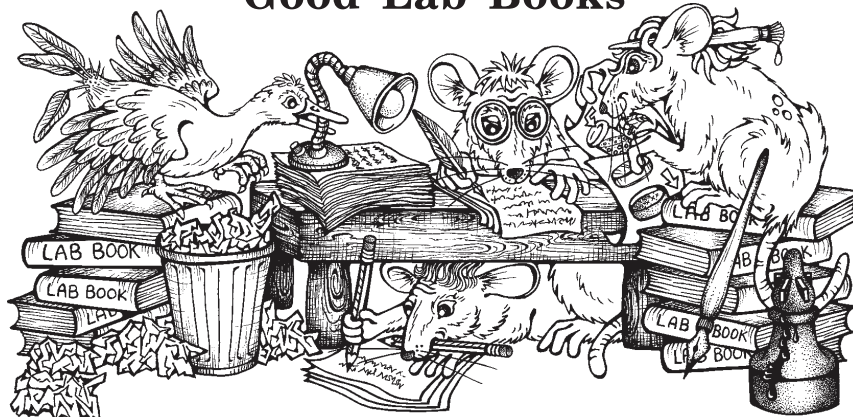
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SETH R. BORDENSTEIN is Assistant Professor in the Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235; e-mail: s.bordenstein@vanderbilt.edu. CHRISTINE BROTHERS is Science Department Head at Falmouth High School, Falmouth, MA 02540. GEORGE WOLFE is Director of the Academy of Science, Loudoun County Schools, Sterling, VA 20164. MICHELE BAHR is Outreach Coordinator of the Bay Paul Center, Marine Biological Laboratory, Woods Hole, MA 02543. ROBERT L. MINCKLEY is Adjunct Assistant Professor and MICHAEL E. CLARK is Research Associate in the Department of Biology, University of Rochester, Rochester, NY 14627. JENNIFER J. WERNEGREN is Associate Professor, Nicholas School of the Environment and the Institute for Genome Sciences and Policy, Duke University, Durham, NC 27708. SARAH R. BORDENSTEIN is Senior Research Specialist in the Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235. WILLIAM S. REZNIKOFF is Director of Education at the Marine Biological Laboratory, Woods Hole, MA 02543. JOHN H. WERREN is Professor in the Department of Biology, University of Rochester, Rochester, NY 14627

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